

# ANNUAL REVIEW OF MICROBIOLOGY

CHARLES E. CLIFTON, *Editor*  
*Stanford University*

SIDNEY RAFFEL, *Associate Editor*  
*Stanford University*

ROGER Y. STANIER, *Associate Editor*  
*University of California*

VOLUME 9

589.95  
An7  
V.9  
set 1

1955

ANNUAL REVIEWS, INC.  
STANFORD, CALIFORNIA, U.S.A.

ANNUAL REVIEWS, INC.  
STANFORD, CALIFORNIA, U.S.A.

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FOREIGN AGENCIES

H. K. Lewis & Company, Limited  
136 Gower Street  
London, W.C. 1

Maruzen Company, Limited  
6, Tori-Nichome Nihonbashi  
Tokyo

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA BY  
GEORGE BANTA PUBLISHING COMPANY

7-0-0

Annual Reviews

42-061-53



7.00

## PREFACE

The preface to this Review provides another opportunity to express appreciation to those who have so diligently and carefully prepared chapters for this volume. The enormous literature in certain fields of microbiology has made it impossible to survey in the space available herein all contributions. Authors have been urged to select those articles that appear to be most pertinent for the development of their reviews and for this reason many worthwhile papers have been omitted from consideration.

The Editorial Committee desires to express its gratitude also to those who have made suggestions for future topics and authors and to invite further suggestions from all workers interested in the development of microbiology.

We regret that factors beyond control have made it impossible to include in this issue the scheduled review of the Neurotropic Viruses by Dr. S. Gard. This delay may prove advantageous in providing time to assess current developments of immunization procedures against the virus of poliomyelitis.

We are pleased to announce that, in co-operation with the National Science Foundation, it will be possible beginning in 1956 to include an annual survey of the microbiologic literature appearing in the Russian language. Dr. Pierre Grabar of the Institut Pasteur in Paris has kindly consented to undertake this enormous task for the benefit of our readers.

At this time we wish to express our thanks to Dr. E. C. Stakman for his efforts on behalf of this Review since its inception. We are pleased to announce the appointment of Dr. S. J. P. Chilton as Dr. Stakman's successor on the Editorial Committee.

Once again we express our deepest appreciation for the valuable assistance and co-operation of the office staff of Annual Reviews, acknowledging in particular the efforts of Mrs. Mary Jean Van Peborgh as editorial assistant, and extend thanks for the co-operation shown by the George Banta Publishing Co.

S.P.C.	J.M.S.
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Volume 10 (1956)

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GENETICS OF BACTERIA AND VIRUSES, *A. H. Doermann*  
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NEUROTROPIC VIRUSES, *S. Gard*  
ANTI-FUNGAL ANTIBIOTICS, *F. Howard, A. E. Prince and B. Champlin*

## ERRATA

### Volume 8

page 31, line 4: *for* realization of the incorrectness of the basis of (his) first criticism underlines the incredibility of DeLamater's interpretation and brings the explanation of the appearances in cocci and bacilli more into line. *read* This realization serves both to underline the incredibility of DeLamater's interpretation and to bring the appearances in cocci and bacilli more closely into line.

page 31, line 13: *for* and states that the cell membranes represent the mitotic configurations described by the author *read* and states that each of his 'chromosomes' can be seen clearly to be the nucleus of a different cell . . .

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## TAXONOMY

By R. E. BUCHANAN

*Chairman, Judicial Commission of the International Committee on Bacteriological Nomenclature, Iowa State College, Ames, Iowa*

Time and space limitations forbid full coverage of the topic "Taxonomy in Microbiology." In consequence, this review will be delimited in several respects. It will concern itself primarily with the bacteria rather than with other groups such as the viruses, fungi, yeasts, algae, protozoa, and slime molds frequently included in microbiology. It will emphasize the problems of nomenclature rather than those of classification and taxon delimitation. It will review only selected publications that have appeared within the last seven years (1948 to 1954 inclusive). It will consider only three groups of topics: (a) some major publications concerning the taxonomy of microorganisms, (b) progress in clarification of rules relating to nomenclature through development of biological nomenclatural codes, with citation of some results, and (c) efforts to stabilize nomenclature in certain taxa. It does not include a catalog of new names of taxa.

### GENERAL MONOGRAPHS

Three publications seem particularly significant; the second edition of the *Dictionnaire* of Hauduroy *et al.* (59), the sixth edition of Bergey's *Manual of Determinative Bacteriology* (10), and the monograph by Zhdanov (106) which illustrates recent Russian thinking on the animal viruses.

There is also the rapidly increasing nomenclatural and taxonomic significance of the journals which abstract the publications in the field of microbiology, particularly *Biological Abstracts*.

In the *Dictionnaire des Bactéries Pathogènes* Hauduroy *et al.* (59) have published a very useful dictionary of pathogenic bacteria. A single alphabetic sequence gives the specific epithets of the species names of bacteria pathogenic for man, animals, and plants, as proposed by their authors, with designation of the species name accepted by Hauduroy *et al.* It contains also the names of the bacterial genera recognized in any one or more of three taxonomic compilations published in 1948, namely Bergey's *Manual of Determinative Bacteriology*, sixth edition (10), Topley and Wilson's *Principles of Bacteriology and Immunity*, third edition (96), and Prévot's *Manuel de Classification et de Détermination des Bactéries Anaérobies* (86). In this latter manual Prévot includes also a synopsis of bacterial genera and other taxa. Hauduroy *et al.* describe all genera with their type species, but only species pathogenic to animals or plants are described. Under each genus the species names of all pathogenic bacteria are alphabetically arranged. There is also appended a symbol indicating the type culture collection, if any, in which the organism is listed as available for distribution. A useful item is

the inclusion under "Classification" of the outlines of the systems of higher taxa and genera as recognized by Prévot, by Breed, Murray, and Hitchens (in Bergey's Manual), and by Wilson & Miles (in Topley and Wilson). Also proposed are 152 new combinations for species names (presumably to be ascribed to Hauduroy *et al.*) together with 22 new names for previously unnamed species. The appendix lists 38 Culture Collections located in different countries, likewise more than 8800 names of species of bacteria, rickettsiae, and viruses catalogued therein.

There are numerous minor errors. For example, the type species of the genus *Nocardia* is given as *N. farcinica* Trevisan 1889; however, this species is not described under *Nocardia* but as *Actinomyces farcinicus* Gasparini 1892. Most species of *Streptomyces* are incorrectly ascribed to Bergey *et al.* rather than to Waksman and Henrici.

The sixth edition of Bergey's *Manual of Determinative Bacteriology* is the most inclusive survey of the classification and nomenclature of the bacteria and the viruses. Each addition gains in prestige as a standard reference volume. The editors would be the first to agree that very much still remains to be done, not only the incorporation of the current advances in our knowledge of microorganisms, but the more difficult task of bringing better order out of the nomenclatural chaos of the past. Most bacteriologists recognize the herculean task that was undertaken and the rapid progress that has been made.

The Bacteriological Code of Nomenclature was internationally approved in 1947. Needed changes were made in the nomenclature of taxa to agree with the rules adopted. True, these rules departed but little from those of the Botanical Code but their application to bacteriology was somewhat uncertain. Some rules were not finally formulated until the meetings of the International Committee on Nomenclature held in connection with the 1953 International Microbiological Congress in Rome. There are, therefore, many names accepted at least tentatively in the Manual whose legitimacy must now be questioned. For example, the following names of higher taxa are illegitimate and incorrect:

*Eubacteriales*, *Eubacteriineae*, *Lactobacteriaceae*, *Enterobacteriaceae*, *Parvobacteriaceae*, *Bacteriaceae*, *Rhodobacteriineae*, *Athiorhodaceae*, *Chlamydobacteriales*, *Chlamydobacteriaceae*, *Myxobacteriales*, *Virales*, *Phytophagineae*, *Zoophagineae*.

These names may either be replaced by correct names or, in cases where a name has been so widely accepted and used as to give rise to confusion and error if rejected, such name should be conserved by international agreement. For example, the family name *Enterobacteriaceae* has been officially and internationally used so commonly that rejection and replacement might well be unwise.

#### RUSSIAN CONTRIBUTIONS ON VIROLOGY

In 1954 Breed & Petrakis (11) reviewed some of the Russian contributions in the field of systematic and nomenclatural virology, and have trans-



lated and published the names and synonyms of species, subgenera, genera, and higher taxa as given by Zhdanov (106) in 1953 in his "Guide to the Human and Animal Viruses." Zhdanov groups all the rickettsiae and viruses into a phylum *Vira* Zhdanov & Korenblut (1949), ignoring the prior use of the name *Vira* by Holmes in 1939 for practically the same taxon. Three classes are included, the *Protophytoviralia*, *Phytoviralia*, and *Zooviralia* for the bacterial, plant, and animal viruses, respectively. The author discusses further the *Zooviralia* only. The order *Rickettsiales* is included under *Vira*. The author has reduced many of the previously published generic names to synonymy with his own proposals. The writer shows a fine disregard for priority, indeed for many of the recognized rules of biological nomenclature. He believes his contribution should lead to the development of a Soviet-approved classification based on the biologic concepts of Michurin. A translation of a part of the preface reads:

The science of viruses, which had its beginnings in Russia, has grown during recent decades into an independent scientific discipline. . . . The need for preparing a Soviet classification and nomenclature of viruses arises from the progress made in the development of general virology and from practical necessity. This need is still greater because a classification has been published abroad . . . developed on a foolish basis, and there is danger that it will be accepted without critical analysis.

The author accepts 34 generic names, of which 27 are new or were published in 1949 by Zhdanov & Korenblut (107). Only six are accredited to other authors. In addition, 14 subgeneric names are proposed, all new. All the names appear to be validly published, but a large proportion are probably illegitimate and must be discarded. In general, the etymology of his new names is acceptable, though there are some errors as in the proposal of the incorrectly formed family names *Strongyloplasmaceae* instead of the correct *Strongyloplasmataceae*, and *Tumefaciaceae* instead of *Tumefaciataceae* from *Tumefaciens*. There are a few errors in the spelling of specific epithets, i.e. the genitive form of *rabies* is *rabies* not *rabii*. This grist of names from Russia will require much sifting, but may well repay the effort.

#### ABSTRACTING JOURNALS

When microbiologists, particularly bacteriologists, are confronted with the need for naming new unidentified strains or organisms under study, to what sources may they turn to make reasonably certain that they do not propose as new a name that has previously been used for another organism and is hence illegitimate? Obviously they first turn to the two compilations whose alphabetic listings and indices are most complete, to Bergey's Manual (10) and to Hauduroy's Dictionnaire (59). However, the contents of these manuals antedate 1948 and many of the monographs of genera in Bergey's Manual are some years older. Until recently there has been no reasonably satisfactory index to current systematic literature in bacteriology. Biological Abstracts now gives promise of filling this need. Until recently it has been rather unsatisfactory due to the lag in publication of annual indices. These

are now rapidly becoming current. The systematic index of each volume lists the names of all genera and higher taxa of systematic interest, and indicates those containing new species. New names of species are readily identified in the subject index. Biological Abstracts is evolving a service to bacteriologists equivalent to that given by Zoological Record to zoologists. Inasmuch as nomenclature in bacteriology is interdependent with nomenclature in botany and protozoology, the systematic indices in these disciplines are also of value in microbiology.

#### CLARIFICATION AND INTERPRETATION OF RULES OF NOMENCLATURE

The distinction between classification and diagnosis of groups, delimitation of taxa, the orderly and systematic management of the groups on the one hand, and the correct naming (nomenclature) of the groups recognized and defined on the other, frequently are not clear in the minds of publishing microbiologists. Bacteriological literature, even the papers published in our best journals, reveals the confusion that exists. Troublesome and unnecessary ambiguities are common.

The giving of names to organisms or to groups of organisms, it has been generally agreed, should be governed by rules that have been internationally approved. Such a set of rules, a so-called code of nomenclature, has been agreed upon to determine what name should be applied to a particular species of organism, or to some higher group, when once the group has been diagnosed and defined. The code assists the microbiologist to determine what the correct name is under a particular set of circumstances. It does not in any way prevent the use of new knowledge in better definition of new groups or of regrouping as may be desired. Only after a determination has been made, at least tentative, of the area to be included within a group, can the rules be of assistance in determining the correct name to be used. There are rules which govern nomenclature but there are not, and cannot be, rules which govern classification of organisms and delimitation of taxa.

Examples of this confusion of nomenclature and diagnosis are abundant. One only is chosen from an excellent review (54) which included the taxonomy of the actinomycetes. The author makes the following puzzling observation:—" . . . it is always permissible to hope that no scheme of classification will be permanently adopted which separates clearly related forms by artificial barriers." There is here the implication in the phrase "permanently adopted" that some agency exists that can legislate or direct the adoption of a classification. Classification of microorganisms must remain fluid, probably indefinitely. There is no agency, in microbiology at least, that can issue authoritative directives for the stabilization of classification. Again this author states:—

The fact that the aerobic nocardias and anaerobic actinomycetes can be sensibly and logically grouped together in *Proactinomyces* (Orskov's Group IIa, and IIb) is admitted but the laws of priority do not permit it. Against such a deadlock it seems impossible to proceed.

Some element of ambiguity is evident in this quotation. No rule or regulation prevents any taxonomist from putting the aerobic nocardias and the anaerobic actinomycetes into one family or into one genus if he believes that the taxa united are sufficiently closely related to justify the reallocation. True, they cannot be united under the generic name *Proactinomyces*. One goes to the Bacteriological Code to determine the rules which fix the correctness of the generic name to be applied under the circumstances. He finds that the rule of priority requires that the oldest legitimate generic name applied to any type species contained in the group is the generic name to be used.

The arrangement of related organisms into groups, the definitions and delimitations of taxa, must always remain fluid. There is no deadlock in any rearrangement that appears logical to an investigator. Every person is free to classify and group as he believes best, remembering always that he must answer for his opinion to his colleagues. The names he applies will be governed by internationally accepted rules, not so his classification.

#### NOMENCLATRURAL CODES, THEIR DEVELOPMENT AND OBJECTIVES

Microbiologists deal with organisms which are alike in that they are small; they are microorganisms. Because of their minuteness, special microbiological techniques have been developed for their study, techniques in general quite distinct from those used for the larger plants and animals. Some of these small organisms are bacteria, some algae, some protozoa, some yeasts, and some viruses. They are usually grouped in part in the plant kingdom and in part in the animal kingdom. Classifications and naming of larger plants and animals antedated those of microorganisms. To prevent undesirable confusion in names, codes of nomenclature were developed, one by the botanists for plants and one by the zoologists for animals. Fortunately the distinctions between the rules and recommendations of the two codes are relatively minor. Basically the codes are alike, although there are many (often seemingly meaningless) differences in details.

Fosberg (57) epitomizes the development and essentials of the Code of Botanical Nomenclature as follows:—

... it was finally realized that in an expanding field of knowledge, order could only be achieved by establishing a set of principles and rules for determining that each plant species would have only one name and that a given name would only designate one species. In 1867, an international congress of botanists adopted a code of rules for the naming of plants, which, though it has been revised several times, is still in effect. From the first, the basic principle in these rules has been that priority of publication is the basis for selection of one from among several names applied to the same plant. More recently has been recognized the obvious principle of determining the application of names by reference to the original (or type) material on which they are based. . . . It is not an over-statement to say that the functioning of the principles of priority and the type method are the main reason that botanists can communicate with each other about plants with no serious ambiguity.

THE INTERNATIONAL CODE OF NOMENCLATURE OF THE  
BACTERIA AND THE VIRUSES

Bacteriologists at their first International Congress in 1930 (84) agreed that the nomenclatural codes of neither the botanists nor the zoologists met their needs. Attempts at liaison with the botanists through the International Committee on Bacteriological Nomenclature were not productive. This Committee was authorized in London in 1936 (91) to develop an independent Code of Nomenclature based upon the Botanical Code, eliminating those provisions applicable only to higher plants, and to include special provisions needed for the bacteria. At the New York Congress in 1939 (95), a Judicial Committee was created and instructed to continue the preparation of a code to be submitted to the next Congress. The code proposed was carefully considered and adopted at the International Microbiological Congress in Copenhagen in 1947.

The microbiologist in his laboratory is concerned with international rules relative to nomenclature formulated by botanists, zoologists, and bacteriologists. He works with bacteria and viruses under the Bacteriological Code, with fungi, yeasts and algae named under the Botanical Code, and with protozoa under the Zoological Code.

The text of the Bacteriological Code was published in English, in both America (19) and England (21), in French (20), in German (23), and in Spanish (22). Some special problems were left unresolved. It was felt desirable to keep the Bacteriological Code compatible as far as possible with the codes of botanists and of zoologists. Both of these groups were in the midst of code revisions. Some of the special problems inherent in the characteristics of the bacteria required further study. Most of the needed revisions of the Bacteriological Code were finally approved at the 1950 Rio de Janeiro (90) and 1953 Rome (92) International Microbiological Congresses. The Editorial Board, appointed in 1950 at Rio de Janeiro, was directed to proceed with editing and final publication.

In both botany and zoology there are large groups of professional systematists working in herbaria and museums, identifying, classifying, and naming plants and animals. In bacteriology the corresponding group is relatively small. But the use of scientific terminology is perhaps even more widespread in the economic and technical literature of bacteriology than is the case in botany and zoology. The classification, and particularly the naming, of the bacteria and the use of their names in publications are not exclusively the province of professional systematists, but of workers who need accurately to identify the tools they are using in pathology, soils, medicine, physiology, biochemistry, genetics, cytology, and many other fields.

The revised "International Code of Nomenclature of Bacteria and Viruses," with amendments and annotations, should be published before the close of 1955. Amendments since the adoption of the Bacteriological Code in 1947, both as proposed (18, 29, 31, 32, 34 to 39, 41 to 44) and as finally

approved (17, 51, 62, 64, 66, 68) have been published, but their significance is not always clear when removed from their context. Many of the amendments were made for the purpose of clarification and frequently to make language more consistent with that of other biological codes. Some amendments are of such immediate interest to all those using the names of microorganisms that a brief review is warranted.

*Name of the Code.*—The official name of the Code was changed from "The International Code of Bacteriological Nomenclature" to "The International Code of Nomenclature of Bacteria and Viruses," with the provision that for a shorter and more informal title "The Bacteriological Code" may be used. While inclusion of viruses has been implicit from the time of the discussion of bacteriological nomenclature at the First International Microbiological Congress in Paris in 1930, it was thought wise to make the inclusion explicit.

*Use of taxon.*—The word *taxon* (plural *taxa*) has been designated (18) as appropriate for the term "taxonomic group" as, for example, a species, a genus, a family, an order, etc. This word first proposed in the botanical code is now approved for use both in botany and in zoology.

*Definition of valid, legitimate, correct name, and name.*—Certain words and phrases employed in systematic biology have come to have special meanings. Recognition of these special uses will prevent nomenclatural confusion. The four terms now more accurately defined in the Bacteriological Code are *valid* (validity of publication), *legitimate*, *correct*, and *name*.

(a). Unless a name has been validly published, it may be disregarded in nomenclature. Whether or not a name is valid depends upon the circumstances of its publication; it must have been validly published. Valid publication requires that the name must have been proposed after 1753 (accepted as the year of the beginning of the Linnaean system of nomenclature), printed material giving name and description must have been sold or distributed to the general public and bacteriological institutions. In other words, it must have been effectively published. Further, the name must have been definitely accepted by its author (publication is not effected by publication of the name as a synonym), and must have been accompanied by a description or citation to a description. A name that has not been validly published is not accepted. The Code states:—"The words 'valid' or 'validly published' as used in these Rules mean 'with standing in nomenclature,' and the words 'invalid' or 'not validly published' mean 'without standing in nomenclature.'"

(b). A legitimate name or epithet is one which has been validly published, does not conflict with any of the rules of the Bacteriological Code, and is therefore available for use. An illegitimate name is one that is not in accordance with the rules.

(c). The correct name of a taxon with a particular circumscription, position, and rank is the name which must be adopted for it under the rules.

(d). A name as used in the Bacteriological Code is any name which has been validly published, whether legitimate or illegitimate.

*Priority of publication as a guide in nomenclature.*—Revised Principle 9 is basic. It states:—"Each order or taxon of lower rank, with a given circumscription, position, and rank, can bear only one correct name, the earliest that is in accordance with the Bacteriological Code." One should always use the oldest legitimate name for the taxon under consideration; this is the correct name. Provision is made for international approval of exceptions which are placed in the official list of conserved names.

*Names of taxa, how formed.*—The names of orders, suborders, families, subfamilies, tribes and subtribes (i.e., of all taxa between subclass and genus) are formed by the addition of the appropriate suffix to the stem of the name of the type genus. Essentially this same rule now holds throughout biology. This means that incorrect names in current use must be replaced, except for some that may be conserved by international action. The revision is a great improvement from the standpoint of logical nomenclature, and, when once the changes necessitated have been made, should simplify teaching.

*Nomenclatural types, their significance in nomenclature.*—Stabilization of nomenclature through the recognition of nomenclatural types has long been the rule in zoology. Acceptance of the type method has more recently been effectuated in botany. The type concept was approved in the 1947 (Copenhagen) Bacteriological Code; in fact, type species of many genera of bacteria were designated in the second decade of the 20th Century. However, adequate procedures for practical use were not finally outlined until the Rome Congress (1953). The pertinent rule states that for every taxon there shall be designated a nomenclatural type. The use of the type concept may best be illustrated by noting its application to the taxa genus and species. For details, reference must be made to the text of the revised code.

The nomenclatural type of genus is the species or one of the species included by the author when the name of the genus was originally validly published. If the author did not designate the type species, then the first subsequent author who fixes the type in accordance with the rules should be followed.

A genus, then, is the type species of the genus together with such other species as the bacteriologist believes to be sufficiently closely related. This definition fails to establish the boundaries of the genus. The fixing of the generic circumscription is a problem for the systematist; the name of the genus, however, remains fixed; in the language of the code "it is permanently attached to the type species."

The second important use of the nomenclatural type concept is in the definition of a species. Throughout biology (botany and zoology), whenever possible, the type of a species is a specimen, a dried plant in an herbarium, a pinned insect in a museum, a bird skin, a fossil, a preserved animal, a skeleton, occasionally an illustration. Nomenclatural types of bacterial species obviously cannot be dried or preserved, non-living, specimens. The Bacteriological Code, therefore, specifies that the type of a species is preferably a



"living culture maintained in a bacteriological laboratory, more particularly in one of the national type culture collections, or it may be a description." Furthermore, the word "culture is to be interpreted as including every method of maintaining organisms in a living state (in a medium, in a host by passage, in cells or exudates, or desiccated)."

A bacterial species may then be defined as the type culture together with such other cultures or strains of bacteria as are accepted by bacteriologists as sufficiently closely related. The designation of type cultures of the type species of the various bacterial genera is a matter of major importance. Here, again, the name is attached to a particular strain. The delimitation of the boundaries of the species is not attempted, the circumscription of the species is the task of the systematist; it is not nomenclature.

Maintenance of such type cultures so that they may be available to workers is one of the principal objectives of a collection such as the National Type Culture Collection in Washington.

Those who describe new species, particularly species which are economically or scientifically important, should deposit authentic cultures in an appropriate type culture collection.

Certain Recommendations which have been included in the Bacteriological Code, if followed by those who describe new species, would be of great nomenclatural and taxonomic assistance to other workers. The author of a new name should clearly indicate the rank of the taxon concerned, designate the type, and, in the case of a species or subspecies, indicate the collection where the type strain or equivalent is preserved. In the publication of a new generic or subgeneric name he should give the etymology of the name, as also that of the specific epithet of a species if the meaning is not obvious.

*Correct spelling of names of taxa.*—Several amendments to the Code suggest methods of determining the correct spelling of the names of taxa, particularly those derived from Greek words and stems. One should conform to classic usage in transliteration to the Latin form. Appendix A of the Code details good precedent for such transliterations. If a later author corrects the spelling of a name or epithet incorrectly transliterated from the Greek, the correctly spelled word may be regarded as a legitimate orthographic variant of the name or epithet.

*Gender of generic names.*—The gender of a generic name determines the correct spelling of the specific epithets of the included species. Rule 28 reads "Generic and subgeneric names which are modern compounds formed from two or more Greek or Latin words take the gender of the last. If the ending is altered, the gender is that of the new ending in the language of origin." Disregard for this old rule has introduced much confusion in the spelling of the names of specific epithets. This means, for example, that generic names which end in *-bacter* are masculine, those ending in *-bactrum* or *-bacterium* are neuter. *Azotobacter agilis* (not *A. agile*), *Acetobacter plicatus* (not *A. plicatum*) and *Achromobacter albus* (not *A. album*) are correctly spelled.

*Organization and functions of the International Committee and Judicial*

*Commission.*—Amendments to the Code, interpretation of its provisions, the formulation and publication of official Opinions, the rejection and conservation of names, these and other duties are assigned to the International Committee on Bacteriological Nomenclature and the Judicial Commission either for initiation of action and preparation of recommendations in some types of cases or for final action in others. The International Committee on Bacteriological Nomenclature consists of not more than five representatives from any single country, nominated usually by one or more of the national societies having to do with microbiology. In the United States the Council of the Society of American Bacteriologists has been responsible for accepting recommendations and submitting nominations. The International Committee is at present made up of nearly 100 members. It is the ultimate authority which passes upon matters relating to nomenclature. The Committee appoints two Permanent Joint Secretaries, one primarily for medical and veterinary bacteriology and one for non-medical.

The Judicial Commission of 12 is elected by the International Committee and, with the two Permanent Secretaries, constitutes a commission of 14 members. The Judicial Commission acts as an executive committee for the International Committee, considers the many requests that come to it for interpretation of the Rules of nomenclature, and issues Opinions relative to the status of names that have been proposed, these Opinions being official unless there is an appeal and the decision of the Commission is reversed by action of the International Committee. The International Committee also created an Editorial Board with instructions to issue at least quarterly a journal which would record the proceedings of the International Committee, the Judicial Commission, the Opinions issued by the latter, reports of the several taxonomic subcommittees of experts, proposals for emendation of the Bacteriological Code, preliminary statements relative to proposals for Opinions, and discussions and original articles relating to taxonomy and nomenclature. The journal developed, the *International Bulletin of Bacteriological Nomenclature and Taxonomy*, is now in its fifth year of publication. It has greatly facilitated communication among the members of the International Commission. Its files should be accessible to all working bacteriologists.

*Subcommittees of Taxonomic Experts.*—Special subcommittees of taxonomic experts are making detailed studies of various taxa of the bacteria, viruses, and other organisms of interest to the microbiologists. The work of these subcommittees is of major significance in the elimination of confusion in nomenclature and taxonomy.

These subcommittees for the several taxa or groups of microorganisms are as follows:

(a). *Viruses.* Dr. C. H. Andrewes, Chairman. This is a relatively large committee, having several subcommissions, as one for the animal viruses, one for the plant viruses, and one for the bacterial viruses. Consideration of the animal viruses is further divided among special commissions of workers.



- (b). *Clostridium*. Dr. A. R. Prévot, Chairman.
- (c). *Enterobacteriaceae*. Dr. E. Hormaeche, Chairman; Dr. W. H. Ewing, Secretary.
- (d). *Leptospira*. Dr. J. W. Wolff, Chairman.
- (e). *Streptococcus and Pneumococcus*. Dr. V. D. Allison.
- (f). *Medical and Veterinary Mycopathology*. Dr. W. J. Nickerson, Chairman.
- (g). *Mycobacterium*. Dr. P. Hauduroy, Chairman.
- (h). *Neisseriaceae*. Dr. E. G. D. Murray, Chairman.
- (i). *Staphylococcus Phage Typing*. Dr. R. E. O. Williams, Chairman.

#### CONSERVATION AND REJECTION OF NAMES

*Rejection.*—The bacteriological Code makes provision for discarding or rejecting names of taxa (usually genera) which, for one reason or another, are regarded as a source of confusion or error. In some cases a name may have been applied to a mixture of bacteria in an impure culture, or based upon an abnormality, or there is uncertainty as to application, or it may be found to be a homonym of the name of a previously named genus of bacteria, plant, or protozoan. A discussion of all the various reasons for rejecting names of taxa cannot be here detailed. Proposals for such rejection are made to the Judicial Commission, which may issue an Opinion, placing the name in the list of rejected names (*nomina bacteriorum rejicienda*). Following is a list of generic names that have thus far been rejected. The reasons for rejection are given in a series of Preliminary Statements by the Editorial Board and in Opinions rendered by the Judicial Commission.

*Astasia* Meyer 1897 (4, 47, 67, 73), *Astasia* Pribam 1929 (4, 47, 67, 88), *Babesia* Trevisan 1889 (4, 46, 48, 65, 67, 102), *Bacteriopsis* Trevisan 1885 (46, 65, 67, 100), *Bacterium* Ehrenberg 1828 (7, 12, 26, 33, 52, 61, 63, 65, 67, 69, 94), *Billetia* Trevisan 1889 (46, 65, 67, 102), *Castellanella* Pacheco and Rodriguez 1930 (4, 24, 47, 65, 67, 81), *Cenomesia* Trevisan 1889 (28, 46, 65, 67), *Charon* Holmes 1948 (4, 47, 60, 65, 67), *Chlorobacterium* Guillebeau 1890 (30, 40, 58, 65, 67), *Coccomonas* Orla-Jensen 1921 (4, 47, 65, 67, 80), *Cornilia* Trevisan 1889 (46, 65, 67, 102), *Dicoccia* Trevisan 1889 (46, 65, 67, 102), *Eucornilia* Trevisan 1889 (46, 65, 67, 102), *Eumantegazaea* Trevisan 1889 (28, 46, 65, 67), *Eupacinia* Trevisan 1889 (46, 65, 67, 102), *Euspirillum* Trevisan 1889 (46, 65, 67, 102), *Leptotrichiella* Trevisan 1889 (28, 46, 65, 67), *Listerella* Pirie 1927 (4, 45, 47, 65, 67, 82), *Mantegazzaea* Trevisan 1879 (46, 65, 67, 99), *Octopsis* Trevisan 1885 (46, 65, 67, 100), *Palmula* Prévot 1938 (4, 47, 65, 67, 85), *Perroncitoa* Trevisan 1889 (46, 65, 67, 102), *Pfeiferella* Buchanan 1918 (4, 15, 47, 65, 67), *Phytomonas* Bergey *et al.* 1923 (6, 47, 65, 67), *Pleurospora* Trevisan 1889 (46, 65, 67, 102), *Pseudospira* Trevisan 1889 (46, 65, 67, 102), *Pseudospirillum* Trevisan 1889 (46, 65, 67, 102), *Rhizomonas* Orla-Jensen 1909 (4, 47, 65, 67, 79), *Rhodospaera* Buchanan 1918 (4, 16, 47, 65, 67).

The rejection of the generic name *Bacterium* Ehrenberg was decided only

after long debate. That the single original (type) species *Bacterium triloculare* Ehrenberg 1828 could not be identified has been recognized for nearly a century. The generic name was applied by later writers to many different groups of microorganisms. One proposal [Breed & Conn (9)] was to recognize it as a temporary genus in which organisms inadequately described could be retained until they could be more satisfactorily placed; another by the Editorial Board (33) suggested that the name be used for a genus to include *Bacterium coli* as named by Lehmann and Neumann (1896) as the type species. Numerous other proposals were studied. The International Committee finally ruled that the name had been used in so many senses that any attempt to perpetuate it as a generic name was unwise, and *Bacterium* was definitely rejected. It was also agreed to reject *Bacteriaceae* as a family name (61).

*Conservation.*—The Bacteriological Code also makes provision for the conservation of the name of a well-established and generally accepted taxon when doubt has arisen as to its legitimacy and replacement might make for confusion.

Fosberg in 1950 (57) pointed out that one of the almost inevitable evils arising from all conservation of names is that it might well constitute an attempt to substitute "decision by authority for taxonomic research." An effort to obviate any such authoritarian procedure is evidenced in the provisions for conservation in the Bacteriological Code. Any one who believes that confusion in nomenclature may be avoided by conserving a name against older homonyms or synonyms may propose such conservation, such proposal to be accompanied by all pertinent background data. All such proposals are published in the International Bulletin of Bacteriological Nomenclature and Taxonomy and discussion or criticism is invited from all interested. The problem is also studied with some care and detail by the Editorial Committee of the Bulletin and, if additional evidence is found, a Preliminary Statement is prepared and published in the *Bulletin*, usually with one or more draft proposals for an Opinion. The problem is then reviewed by the Judicial Commission. An affirmative vote of at least eight members of the Commission is required for approval of an Opinion. If objection is filed protesting the decision of the Opinion, this objection is also published, and final determination made by the International Committee.

The following generic names of bacteria have been conserved (placed in the list of *nomina generum conservanda*):

*Beggiatoa* Trevisan 1842 (65, 97, 98), with type *Beggiatoa alba* (Vaucher) Trevisan 1845 (*Oscillaria alba* Vaucher 1803) (103); *Gallionella* Ehrenberg 1838 (53, 65), with type *G. ferruginea* Ehrenberg 1838; *Klebsiella* Trevisan 1885 (65, 100), with type *Klebsiella pneumoniae* (Schroeter) Trevisan 1887 (101); *Kurthia* Trevisan 1885 (65, 100), with type *Kurthia zopfii* (Kurth) Trevisan 1885 (100); *Listeria* Pirie 1940 (45, 83), with type *Listeria monocytogenes* (Murray, Webb & Swann) Pirie 1940, against *Listeria* Necker 1790 (77); *Neisseria* Trevisan 1885 (65, 100), with type *Neisseria*

*gonorrhoeae* Trevisan 1885; *Pasteurella* Trevisan 1887 (65, 101), with type *Pasteurella cholerae-gallinarum* Trevisan (101); *Nocardia* Trevisan 1889 (65, 102), with type *Nocardia farcinica* Trevisan.

Several draft proposals for conservation of generic names have been published for consideration by the Judicial Commission but have not yet been acted upon. These proposals concern names of genera and species which are in common use, but whose correctness has been questioned. Discussion by those interested is desired by the Judicial Commission, whose Editorial Board will be pleased to receive comments and criticisms of proposals. Among these proposals are *Aerobacter* Beijerinck 1900 (5, 26) to be placed in the list of conserved names of genera with type species *Aerobacter aerogenes* (Kruse) Beijerinck 1900; *Escherichia* Castellani and Chalmers 1919 (24, 26), to be placed in the list of conserved names of genera with the type species *Escherichia coli* (Migula) Castellani and Chalmers 1919.

Some misunderstanding has arisen as to the exact meaning and effect of conserving a generic name. The Editorial Board of the International Committee (49) in a Preliminary Statement comments as follows:—

In any consideration of these proposals, the limitations of the term conservation should be kept clearly in mind. . . . The generic name *Klebsiella* published in 1885, the name *Escherichia* published in 1919 and *Aerobacter* published in 1900 are proposed for conservation. Each has its type species designated. Should an author conclude that the differences between the genera *Klebsiella* and *Aerobacter* or between *Aerobacter* and *Escherichia* are not sufficient to justify their segregation, he would be justified in uniting the two genera and using the name of the genus having priority in spite of the conservation of the second generic name.

In other words, a genus with a conserved generic name may be merged with another genus whose generic name has priority if the two genera have different type species.

The generic names *Micrococcus* Cohn 1872 and *Staphylococcus* Rosenbach 1884 have long been used rather interchangeably in the writings of bacteriologists. Should one genus or two be recognized? If two genera are recognized, how may they be differentiated? If one genus is recognized, should it be called *Micrococcus* or *Staphylococcus*? Can the type species *Micrococcus luteus* be recognized as described by Cohn? Breed (8), Cowan, Shaw & Williams (27), Gibson (57a), and Shaw, Stitt & Cowan (93) have all made proposals. A Preliminary Statement (1954) (50) has been published outlining the problem. Apparently the correct answer must await completion of studies now under way in several laboratories.

Following studies by committees of experts, and upon their recommendation, Opinions have been issued in a few cases conserving the names of species in order to stabilize the nomenclature. The species name *Streptococcus agalactiae* Lehmann and Neumann (72), for the organism associated with bovine mastitis, has been conserved against synonyms having priority.

Opinions have been issued approving the recommendations of the *Enterobacteriaceae* Subcommittee of the International Committee to con-

serve the species names *Shigella flexneri* Castellani and Chalmers 1919 (24, 67) for the species first described as *Bacillus dysenteriae* Flexner 1900 (56), *Shigella boydii* Ewing 1949 (55, 67) and *Shigella sonnei* (Levine) Weldin 1926 (67, 104).

*Abbreviations of generic names.*—A question frequently asked of, and by, editors of journals in the field of bacteriology is "What is the correction abbreviation for the generic name——?" Cowan (25) concludes that bacteriologists should conform to biological custom and use the first letter as an abbreviation. A questionnaire to editors to 77 English-language journals with 85 per cent response revealed that, although several American medical journals use abbreviations as given in Dorland's Medical Dictionary, the most common journal practice is to write the generic name in full "when first mentioned (a) in the title, (b) in the paper, and (c) in the summary, with subsequent use of a single (initial) letter for the generic name." Where there is use of two generic names with the same initial letter, the generic name should be spelled in full if there is danger of ambiguity. The futility of attempting to give to each genus a characteristic abbreviation is evidenced by the fact that at least seven generic names have been proposed having *Strepto*- as the first six letters and that *Thio*- is the prefix of 27 generic names.

*Use of generic names in the vernacular and in the plural.*—When the name of a genus must be used repeatedly in a publication, it is often wise to use the vernacular or "common" name. Such use tends to reduce stilted phraseology. Good use of this device is to be found in Erikson's (54) review of the *Actinomycetales*. For example, in a discussion of species of bacteria belonging to the genus *Nocardia*, she uses "saprophytic soil nocardias," similarly "soil streptomycetes," "other mycobacteria," "anaerobic actinomycetes." The use of the generic Latin name in the plural is also permissible, and for such use there is ample good precedent. For example, the genus *Nocardia* is made up of its type species *N. farcinica* together with such other species as bacteriologists regard as sufficiently closely related. One may say "This is a species of *Nocardia*." However, custom has sanctioned the use of the equivalent statement "This is a *Nocardia*." Where two or more species of the genus are under discussion, it is permissible to say "These are *Nocardiae*." There are those who have contended that, inasmuch as under the nomenclatural code there can be but a single genus with a given name, the use of the generic name in the plural implies the existence of two or more genera. Long usage and custom have decreed otherwise.

The vernacular equivalent of the generic name may be formed variously. The exact spelling of the Latin name may be used; in some cases it is advantageously modified. The generic names *Nocardia* and *Salmonella* become nocardia and salmonella. However, in many generic names the complete stem is not included. For example, the generic name *Pseudomonas* in the vernacular may be either pseudomonas or pseudomonad. Perhaps pseudomonad is preferable as it contains the entire stem of the word, and its

English plural pseudomonads is simpler than pseudomonades, the plural of pseudomonas. The English equivalents of generic words ending in *-myces* may end either in *-myces* or *-mycete*, the plural of both ending in *-mycetes*. Generic names ending in *-us* or *-um* customarily retain the Latin plural as in *lactobacillus* (*-i*) and *mycobacterium* (*-a*). Prince *et al.* (89) appropriately use as the title of a paper, "*The Flavobacteria*." Some generic names of Greek derivation end in *-ma* as *Treponema*. If one chooses to anglicize in the form *treponema*, the plural should be *treponemata*, never *treponemae*. The common treatment of such Greek words is to change the last component to *-neme* and use the standard English plural, *i.e.*, *treponeme*, *treponemes*. ("the Reiter treponeme"). In anglicizing generic names ending in *-a*, one may use either the English or the Latin form. For example, Erikson (54) uses the English plural "*nocardias*" and the Latin plural "*micromonosporae*." Traditionally the use of the Latin plural is perhaps preferable.

#### REPORTS OF TAXONOMIC SUBCOMMITTEES

Space limitations will not permit review of the progress reports of the several taxonomic subcommittees of the International Committee on Bacteriological Nomenclature (1, 2, 3, 13, 14, 70, 71, 74, 75, 76, 78, 87, 105).

The *Enterobacteriaceae* Subcommittee had its origin in the recognition of the practical significance of utilizing serological techniques in the better identification of strains of enterobacteria responsible for disease production in man and animals. Such identifications have proved of particular significance in epidemiological studies, for example, on outbreaks of food poisoning. Kauffmann's laboratory in Copenhagen was recognized by the World Health Organization as the first international center for identification of salmonellae by all techniques but with special emphasis upon the adequate characterization of serotypes and methods adapted to practical field use. Dr. Kauffmann associated with himself a group of specialists which was recognized as the *Enterobacteriaceae* Subcommittee of the International Committee on Bacteriological Nomenclature. Later Dr. Edwards of the U. S. Public Health Service was co-chairman during a period of active development. Dr. Hormaeche of Uruguay is now chairman with Dr. W. H. Ewing of Atlanta, Georgia, secretary. The area of study broadened quickly to include serotype characterization of strains of bacteria belonging to many species and genera of the *Enterobacteriaceae*. It is manifestly impracticable to review nomenclature and taxonomy as found in many scores of papers whose publication has been sponsored or stimulated by the work of this subcommittee. Two comprehensive reports have been issued on the so-called "groups" (usually genera), *Salmonella*, *Arizona*, *Bethesda*, *Ballerup*, *Escherichia*, *Alkalescens-Dispar*, *Klebsiella*, *Proteus* and *Providencia*. The basic problems of diagnosis are discussed and the diagnostic antigenic schemata outlined. These reports are most valuable summaries for students, teachers, and diagnosticians.

The *Enterobacteriaceae* Subcommittee has two nomenclatural and taxo-

nomic goals. One is to produce a workable scheme of characters that will enable the bacteriologist who requires highly definite identification to recognize the organism with which he is dealing, usually because such recognition is essential in important epidemiological studies. This goal is certainly in sight. In most cases the worker can place the organism in its proper pigeon hole and has a code number which he can use in any discussion of it. The subcommittee is to be commended for this accomplishment.

The second goal is also important, but its achievement is not so clearly in sight. The extraordinary array of serotypes must eventually be so arranged and classified that they will fit into taxa of the more conventional kind. True, the situation is complex, but not more so than may be found in many other areas of systematic biology. Some progress has been made towards better differentiation of species and genera. A usable scheme which can be incorporated into systematic treatment of bacteria with adequate distribution of serotypes under species would prove most helpful and is attainable. The prestige and usefulness of the subcommittee will be much enhanced if there can be emphasis upon the attainment of this second goal.

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## MORPHOLOGY OF VIRUSES<sup>1</sup>

BY FREDERIK B. BANG

*Department of Parasitology, The Johns Hopkins University, Baltimore, Maryland*

The Microbe is so very small  
You cannot make him out at all,  
But many sanguine people hope  
To see him through a microscope.  
His jointed tongue that lies beneath  
A hundred curious rows of teeth;  
His seven tufted tails with lots  
Of lovely pink and purple spots,  
On each of which a pattern stands,  
Composed of forty separate bands;  
His eyebrows of a tender green;  
All these have never yet been seen—  
But Scientists, who ought to know,  
Assure us that they must be so . . . .  
Oh! let us never, never doubt  
What nobody is sure about.<sup>2</sup>

The shape and structure of some viruses are now sufficiently understood so that some systematic treatment is possible. We have concentrated in this review on the viruses infecting vertebrates and refer only in pertinent places to the morphology of viruses parasitic upon plants, bacteria, or insects. The last group has been well reviewed by Bergold (1, 2). A variety of factors influence the size, shape, and appearance of biological material when prepared for electron microscopy. These have been carefully considered by Robley Williams in two recent reviews on viruses (3, 4). We will, therefore, not emphasize them here, but will concentrate more on the biological data available for each group of viruses. The importance of the shape of an infectious agent has been emphasized recently by Andrewes (5) who in a series of proposals for the classification of viruses has listed the morphology of the agent as the first criterion. This is embarrassing when little or nothing is as yet known about a particular agent, and difficult when, in a series of uncritical reports, coated smudges on a collodion screen have been labeled as the virus.

In the absence of growth of viruses on nonliving media, it is impossible to be sure that the agent has been completely separated from host material, and it is logically impossible to know whether one is dealing with the complete virus. However, as with malaria parasites and leprosy bacilli, strong circumstantial evidence as to its morphology can be obtained. We believe that this evidence may best be assessed by classifying it under a series of

<sup>1</sup> The survey of literature pertaining to this review was completed in March, 1955.

<sup>2</sup> Reprinted from CAUTIONARY VERSES by Hilaire Belloc, by permission of Alfred A. Knopf, Inc.

criteria. These may be fulfilled in varying degrees. They are presented in Table 1 and are an extension of those used originally in identifying the Newcastle disease virus (6).

CRITERIA FOR THE IDENTIFICATION OF A VIRUS WITH PARTICLES  
SEEN BY MICROSCOPY

*Characteristic Appearance.*—(a) As Individual Particles: Shape and resistance to change of shape. Appearance under specified conditions or following enzymatic treatment. (b) As Groups of Particles: Particularly in tissues either in cultured cells or thin sections.

*Association of Agent with Disease or Infection.*—(a) Extract of Infected Tissue as Compared with Various Controls. (b) Comparison of Sections or Tissue Cultures of Infected with Normal and Damaged Tissues.

*Physical Testing of the Association of Particles with the Infectiousness of Preparation.*—(a) Centrifugation. (b) Filtration. (c) "Analytical Electron Microscopy."

*Association of Particles with Other Known Activity of the Virus.* (a)—Adsorption and Elution from Red Cells. (b) Physical Testing as under 3 Applied to Hemagglutinating Activity or in some Cases Complement Fixation.

*Agglutination of the Particles by Immune Sera, Preferably Sera from Convalescent Animals.*

*Infection Produced by a Very Few Particles.*

Only in a few instances have all of these criteria been fulfilled. Perhaps the most completely studied is the virus of vaccinia which was identified before the use of the electron microscope (7). Thus the recent work on the morphology of this agent has increased meaning. Some particles may be considered as identified as virus without all criteria being applicable. For instance, tumor viruses may produce tumors only when many particles are placed on the skin and thus the last criterion cannot be fulfilled. It is reasonably certain that the tobacco mosaic virus has been identified, and yet it takes many particles rubbed on a leaf to initiate the infection. This is presumably due to insufficient exposure of cells.

Each of the criteria listed is a complex one, and varying judgments as to the completeness with which these criteria have been met will be made. In Table I we have listed the different viruses which have been studied as to form and structure, and have included references to work contributing to the evidence in favor of the association of particle with virus. Individual blocks in which no references are made may be blank because this reviewer has missed a particular paper with evidence, but such is the paucity of our knowledge that most of the blank spaces are due to the absence of data. Certain viruses have not been included in these tables because it does not seem to this reviewer that any evidence at all has been produced which would allow one to identify or characterize the particular virus which presumably has been studied in the electron microscope.

TABLE I

CRITERIA FOR IDENTIFICATION OF A VIRUS WITH PARTICLES SEEN BY MICROSCOPY

	Morphology	Association with Disease	Association with Infection by Physical Means	Association by other means	Infection by few Particles	Agglutination
Psittacosis	2, 10, 21	2, 10, 18				10
Ornithosis	21					
Felinosiis	21, 121					
Meningopneumonitis	13, 21, 122	12, 13	122			
Murine pneumonitis	19, 21		19			
Lymphogranuloma venereum	8, 9, 21, 122	8, 9, 122				
Fowl Pox	22, 26, 30	22, 30				
Variola	30, 31, 41	30, 37, 41	7, 23		7, 23	7, 23, 33
Vaccinia	7, 23, 37, 43, 46	7, 23				
Ectromelia	37, 50	37, 50				34
Myxoma	42, 43					
Molluscum contagiosum	19, 37, 47, 50, 51	50, 51	19			
Herpes zoster and Chicken Pox	50	50				52
Herpes simplex	54, 56	53, 54, 56	53			
Influenza Spheres	50	50	57, 58, 66	59, 60		62
Filaments	63	4, 63, 64, 65	66	63, 66		
Fowl plague	85, 87	85, 86	59, 85, 89			
Newcastle	6, 69, 70, 71, 72	6, 69, 70, 71, 79	6, 69, 70	6, 69, 80	6, 69	6
Mumps	84	84	84	84, 123		
Tumor Viruses Rous Tumor	90, 91	90, 91				
Mammary Tumor Initiator of Mice	94, 95, 96	94, 95, 96				
Rabbit Papilloma	99, 100		99			
Human Papilloma	101, 102, 103	101, 102				
Avian Leukosis	76, 107	76, 107	105c	105a, 105b		
Lymphomatosis	106	106				
Encephalitis Equine Encephalomyelitis	108, 110, 112	108, 110, 112	108			
Encephalomyocarditis		114				
Poliomyelitis-like Viruses Cocksackie	113	113				
Poliomyelitis I		115, 116, 124	115, 116, 124			
Poliomyelitis II	117	116	116			
Poliomyelitis III		116	116			
Theiler's Virus	120	120				

## PSITTACOSIS—LYMPHOGRANULOMA VENEREUM GROUP

Knowledge of the morphology of this group of agents dates back to the original careful descriptions of the "L.C.L." bodies present in the two diseases for which the whole group of agents has been now named. It should be remembered that, because of extensive study by light microscopy of these particles as they formed in the infected cell either in the host or in tissue culture (2, 8, 8a, 9), one can say that the first two criteria in our table have been fulfilled.

Electron microscopy of these viruses has until very recently dealt with isolated particles and in most cases has been merely a description of the appearance of the elementary bodies. Heinmets & Golub (10), however, studied both normal and infected chick chorioallantoic membranes under different conditions of fractionation and suspensions of psittacosis elementary bodies which were obtained from the allantoic fluid. The latter were agglutinated by antisera prepared against the same virus from another source. It is not clear, however, whether the sera shown to agglutinate the particles may have had antibodies to chick material. Two studies of the association of infectiousness with the elementary bodies have been reported. In one (Crocker 1954), a comparison of the number of particles presumably characteristic of meningo-pneumonitis was made with the infectiousness of several preparations. The  $LD_{50}$  for embryos was calculated to contain an average of 200 to 1000 particles. The number of particles necessary to initiate infection ( $ID_{50}$ ) was however about 0.6 log less. In the other study (Gogolac 1953) the number of elementary bodies as seen by light microscopy and counted by a modification of the Williams & Backus method (11) was correlated with the infectiousness of the preparations during purification.

Thus there is a moderate amount of evidence, much of it carried over from light microscopy, that the elementary bodies of these infections are the virus particles themselves. The problem becomes more difficult, however, when it is recognized, particularly in preparations of virus obtained from allantoic fluid, that there are two general classes of sizes to these particles—one ranging from 300 to 440  $m\mu$  which has been found by most workers who have studied dried preparations in the electron microscope, and the other from 650 to 800 (10, 12). The identification of the larger and more diffuse sacs which have no distinctive morphology, as part of the virus cycle, is much more difficult. These larger forms may correspond in some way to the "plaques" which have been described previously by light microscopists, and Gaylord's studies (13) of sections of infected membranes show such large sacs within the infected cells. However, sac-like extrusions from the cell are seen in other virus infections of the chorioallantoic membrane (14) and probably are an accompaniment of degenerating cells (15). It seems clear that the electron microscope studies of isolated particles have not yet furnished a differentiation between larger forms of virus and degeneration

blebs. Thus we cannot adequately indicate the sequence of events proceeding from one type of particle to another.

The most direct evidence is that obtained from sections of tissues. Gaylord (13) has published excellent electron micrographs showing the elementary bodies in the chorioallantoic membrane. Since these preparations were made by dropping the membrane before infection, damage of cells occurs both following the separation from the shell membrane (16) and due to the infection itself. However, the pictures do indicate that "the virus can multiply by binary fission or by multiple endosporulation and that elementary bodies are a spore-like stage." Similar budding forms of feline pneumonitis virus have been found in sections of mouse lung by Karrer (17).

In a study (17) combining light microscopy, serology, and tests for infectivity, Bedson & Gostling (18) found that the large forms or initial bodies are first found 8 hr. after the infection of mice. The forms occurred singly or in pairs. All of 16-hr. infections showed the large form. They believed that "psittacosis virus multiplies by binary fission."

Occasional doublets are seen in preparations of elementary bodies (19), but it is clear that the meaning of these forms will be settled only by a series of studies at set time intervals, perhaps using large inocula on tissue culture cells, and by a careful light and electron microscope study of these in series.

The diameters of the dried and collapsed particles studied in direct preparations made from saline or water suspensions are greater than the particles in sections (13) or prepared more carefully (20). The diameter of the virus (elementary body) is probably closer to 250  $\mu$  rather than the 350 to 450  $\mu$  usually obtained with dried preparations (12, 21). It seems from both the dried preparations and the sections (13, 17) that the smaller virus particles have a much denser center and a diffuse or less dense periphery.

#### POX VIRUSES

The availability and size of the pox viruses have made them attractive subjects for study in the electron microscope. Evidence associating the individual elementary body of fowl pox (Goodpasture) with the infectious agent was based on light microscopy and microdissections (22). Five of the six criteria here used for the identification of the elementary bodies of vaccinia with the infectious particle were established by Rivers *et al.* (23).

The first electron micrographs of such particles (24) showed these particles to be roughly brick shaped, and to have a complex internal structure and an apparent outer membrane. The application of a technique of peptic digestion by Dawson & McFarlane (25) showed that many particles had a dense central portion which was resistant to this digestion. Incubation of these particles with purified pancreatic deoxyribonuclease caused a loss of density of the central body. "These observations show that this body has some at least of the characteristics ordinarily attributed to a nucleus, and seem to justify the use of this term for it." Since then there have been a var-



iety of studies of the group and for clarity's sake we will discuss each member of the group individually. It is not clear, however, whether there is a significant difference between the members.

*Fowl Pox.*—This virus is usually considered as larger than the other members of the group (26) but has been studied in the electron microscope less than the others. It occurs within a lipoid inclusion in both the infected chicken skin (22) and in the infected chick embryo chorioallantoic membrane (27). Thin sections of these inclusions studied by electron microscopy show the elementary bodies within the inclusions (28 to 30a). Lepine *et al.* (31) have previously shown that digestion with pepsin brought out the central dense area, and suggested that there were similarities between the central "nuclear" areas and the peripheral cytoplasm of these particles and bacterial cells. The intact virus particles showed little internal structure, and were resistant to papain and trypsin. A differential digestion was obtained by Bang *et al.* (29) following exposure to pepsin. Excellent and provocative pictures of the virus in sections of chick chorioallantoic membranes have been recently published by Morgan and co-workers (30). Since, however, they state that the viruses of vaccinia and fowl pox "were of similar structure and size, averaging  $200 \times 300 \text{ m}\mu$  with considerable individual variation," we will discuss this more fully in connection with the studies on vaccinia. It is apparent that extensive electron microscope studies should be carried out on strains of virus which are clearly identified and which should be checked for identification after the study. No identification of this strain of fowl pox is presented in this paper.

*Vaccinia and Variola.*—The first phase of the electron microscope study of these viruses was the direct description of material isolated from experimental infections (17, 25, 26). The morphology of these particles, particularly as they occurred in clumps from the tissue, has been considered so characteristic that differential diagnosis of small pox and chicken pox has been proposed on the basis of electron microscopy (10, 32). In our experience with vesicle fluid from cases of chicken pox the difference in size of the two types of elementary bodies does seem apparent, but the accuracy of the test is not yet established.

Of the six criteria for identification of a virus, agglutination by immune sera is the least satisfactory one for our purpose. Vaccinia virus has been agglutinated by immune sera [Rivers (23)] and this agglutination studied in the electron microscope (33). However, the sera were not obtained from an animal in which the only antigenic stimulus was the virus which multiplied in that animal. Thus the agglutination of these particles is not in itself strong evidence of the virus nature of the particles. Agglutination of ectromelia virus by immune guinea pig sera but not by immune mouse sera was reported by Jahn in 1939 (34). Again this is not proof of the virus nature of the particle.

Despite the indefiniteness of the immunological data, the evidence is very good that the characteristic brick-shaped particles seen in vaccinia



infections are the virus particles. Subsequently localization of these particles within the cell has been studied and attempts made to interpret the development of the particles. Recent improvements in thin sectioning techniques have produced increasingly better pictures (30, 35, 36, 37). In all it is clear that the individual virus particles are finally disseminated through the cytoplasm. It was early suggested that some particles were found either inside or closely associated with mitochondria (35). This is not apparent late in the infection of the cell, and careful serial studies of the early infection have not been reported. It is interesting to note that a plant virus, i.e., beet yellows virus, has been found closely associated with the chloroplasts of the leaves (38).

Gaylord & Melnick's study (37) of several members of the group both in the infected chick embryo and on the cornea of rabbits was the first to show clearly the internal structure of the particles as they exist in the tissue itself, and opened up the possibility of following changes in morphology by this means. They describe a variety of particles, including hollow spheres, some with dense centers, others with a complex internal structure resembling bars or dumbbells. They suggest that development proceeds from hollow spheres towards a dense solid particle. Particles with the dense centers are considered as intermediate. No time sequence studies are offered in support of this proposal, and the studies were apparently limited to infections which were 72 hr. old for the chick membrane and 96 hr. for the cornea. In an infection of this age a great deal of virus multiplication has taken place.

Undoubtedly the most striking pictures so far published are those of Morgan *et al.* (30) and their pictures are worth repeated study. Again, however, no study over a period of time of infection is reported and thus it was not possible to follow the actual development of the final morphology. The nuclear area of the virus is clearly shown, and in some cases a less dense area is present between the "nucleoid" and the "viroplasm." Viral particles lying outside the cell, thus presumably extruded from the cell, showed disc-shaped inner bodies and double limiting membranes. Serial sections show the dense nucleoid present in one section and absent in the next section of the same virus particle. Thus the exact localization of the "nucleoid" is difficult to determine in one section.

With the increased resolution afforded by the study of extremely thin sections, problems of artifact production and determination increase correspondingly. Recent work (39) on polymerization damage which occurs during the process of embedding tissues in methacrylate would suggest that the "incomplete membranes surrounding the component of viral structure" are incomplete because of the damage and destruction to cellular structures which occurs at polymerization. This damage has a higher degree of localization and makes it difficult to differentiate between artificially "exploded" virus particles and developing forms. Since these particles in the cell fail to show the brick-like shape, well recognized in dried preparations, the authors suggested that the latter are artifacts of drying.

Localized swelling in tissues moreover would make it difficult to determine whether the true shape of the individual particles is brick-shaped or whether the lack of such forms in the embedded material is due to swelling during polymerization.

Peters & Masemann (40, 41) have made direct press preparations of vaccinia virus on the surface of the infected chorioallantoic membrane and rabbit corneas and transferred them to collodion screens. Such preparations have certain advantages over thin sections, even if a similar high resolution is not obtained. A more correct distribution of size measurements may be obtained. Their measurements showed "clearly that the length and width distribution did not follow a normal Gaussian curve" (40). The length was with few exceptions between 240 and 380  $m\mu$ , the width between 170 and 270  $m\mu$ . These observations and the fact that the greatest length (about 360  $m\mu$ ) corresponds to double the lowest width (about 180  $m\mu$ ) appeared to these authors as a strong argument for the acceptance of the idea of division (Querteilung).

The structure of the virus was determined at different times of infection following digestion with pepsin at pH 1.95–2.05 and subsequent fixation with formalin and alcohol. Both ring and double forms of elementary bodies were illustrated. Not all the viruses were digested by the treatment. Good differentiation of structure was obtained by 30 min. treatment with .02 per cent solution and no further changes were obtained with treatments from 2 to 24 hr. By arbitrarily classifying the different appearances of the elementary bodies after such digestion into four different groups, the authors followed the changing proportions of different forms. The classes were: (a) Intact elementary bodies; (b) Beginning digestion; (c) Clearly differentiated inner body and membrane; (d) Membranes without distinct inner bodies. The proportion of these forms was not affected by the length of treatment with pepsin. A greater proportion of the type *c* and *d* forms was obtained on removing an infected rabbit's eye two days after infection, and placing the eye at incubator temperatures (105°) for 4 hr. thereafter. The same was done with dead embryos. The authors believe it possible that the inner body is a nucleus in a perinnuclear area, and point out that chromosomes of higher forms are also resistant to pepsin. The presence of ring forms and double forms within the elementary body is similar to the "nuclear" forms seen in bacteria.

*Myxoma*.—Morphological studies of myxoma have shown that this virus is very similar in size and shape and internal structure to the other members of the pox group. This has been apparent both in sections (42), and in purified preparations (43).

The fibroma of rabbits described by Shope has a certain immunologic relation to the myxoma virus, and even may be converted into such by appropriate treatment. The fibroma virus has an appearance similar to the viruses of myxoma and the pox group as seen in suspensions and in thin sections of rabbit tissue (44, 45).

*Structural Similarities to Bacteria.*—In further studies using the combined electron microscope and enzymatic method of analysis Peters *et al.* (46, 47, 48) have obtained more specific data relating the "nucleus" of the virus particle to a specific DNA containing mass. It was first shown (48) that bacterial nuclei could be digested by the combined application of first ribonuclease, then pepsin, then deoxyribonuclease which presumably depolymerizes the DNA but does not remove it from the cell. The final application of pepsin removes the nuclei. The advantages of using Chabaud's fixative are emphasized.

A similar digestion of the nuclei (46) of vaccinia virus from chick membranes was obtained by first treating with pepsin, then following by DNA (pancreatic) or streptodornase, and finally by treatment with pepsin again. This results in large numbers of apparently empty membranes. These resemble empty walls of bacteria. The authors call attention to the structural analogies between the pox viruses and bacteria (49).

*Molluscum contagiosum.*—It has been early demonstrated [see Ruska for review (50)] that particles very similar in shape, appearance, and size to the viruses of vaccinia and ectromelia could be obtained from the skin lesions of *Molluscum contagiosum*. These have been studied further in thin sections of such lesions by several workers (37, 51). An extensive enzymatic analysis (47) of the elementary bodies obtained from the lesions of this disease showed that these particles also have a dense center, presumably a nucleus, that may be digested out by the combined use of deoxyribonuclease and pepsin, leaving a series of empty membranes behind. The relationship between the membranes left after digestion and the incomplete membranes seen in sections has not been established. An accurate description of the morphological structures involved in the reproduction of the pox virus is not yet possible.

*Herpes zoster and Chicken Pox.*—Little has been added to the description of the suspected virus particles since Ruska's review. Agglutination of the elementary bodies (virus particles) by human convalescent sera was observed with the light microscope more than 20 years ago by Amies (52). A careful series of sections through the skin lesions of chicken pox would be valuable.

*Herpes simplex.*—Although this virus has been a standard laboratory model for many years, only recently has the morphology of the virus itself been studied. This has probably been due to its smaller size and to the fact that it has not been possible to obtain concentrated and purified preparations. In one attempt (53) in this direction, advantage was taken of the growth of the virus following allantoic sac inoculation. Material, concentrated and partially purified by three centrifugations, showed particles about 116  $\mu$  in size. The published pictures are few and not convincing of a characteristic morphology. They agree with previous pictures of particles in fluid obtained from herpetic lesions.

An excellent study by Morgan and co-workers (54) of thin sections of the chick chorioallantoic membrane infected with a laboratory strain of

herpes shows many clusters of characteristic virus-like particles both within the nucleus and outside in the cytoplasm. Many of these show a dense center and many have a well-demonstrated double membrane around them. It is difficult, however, in such studies to trace the development of the particles and, although highly characteristic groups of particles with dense central areas and lighter peripheries were found, the smaller (30–40  $m\mu$ ), less sharply defined particles, which the authors refer to as primary bodies, are much more difficult to identify. Some of the particles presented are similar to the "virus" particles found in 15 per cent of tissue cultures of chick embryos (55, 55a). Since this strain of herpes virus has been carried through a number of embryo passages since 1947, the possibility of contamination by chick embryo viruses needs to be considered.

Reissig & Melnick (56) have studied a closely related virus (herpes B) in tissue cultures of monkey kidney cells. By infecting at a given time in a way in which most of the cells are exposed to virus it was possible to follow the appearance and development of the lesions in the cells and the particles within them. They find the same groups of particles with dense centers, as described by Morgan *et al.*, but observe them appearing in the cytoplasm and the nucleus at the same time. They classify them as (a) particles with one membrane which range in size from 60 to 100  $m\mu$ , (b) those with two membranes (120 to 180  $m\mu$ ) and which occur both in the cytoplasm and the nucleus, and (c) particles about 180  $m\mu$  in diameter but with a double central body. These last were found both in the cytoplasm and on the external surfaces of the cell. More valuable information concerning the structure of the herpes group of viruses has thus been furnished by a study of the infected tissue itself rather than by a study of suspensions of the agent.

#### INFLUENZA, MUMPS, NEWCASTLE GROUP OF VIRUSES

*Influenza.*—The first indication of a complex situation was the discovery by Mosley & Wyckoff (56a) that filaments of the same diameter as the virus were to be found in the allantoic fluid. Later it was found that recently isolated strains commonly gave rise to long filamentous forms in the allantoic fluid, whereas the embryo-adapted strains yielded spherical forms. Tissue culture studies of infected membranes showed the two forms to occur in the same culture (14).

*Identification of Spherical Forms as Virus Particles.*—The original work on the purification and size determination of these particles is well-summarized in Ruska's review (50). As part of an intensive study during World War II it was shown that infectivity was clearly associated with particles (57, 58). In contrast to the pox viruses it has not been possible to separate the hemagglutinin activity from the infectious particle. Since Dawson & Elford's demonstration (59) in the electron microscope of the adsorption of the spherical particles on to the surface of laked red blood cells, a number of pictures have been published showing varying amounts of virus on the cells.

A direct proportion between the virus concentration and the number of particles adsorbed from a suspension of inactivated virus has been recently demonstrated (60).

Although Knight (61) showed that precipitin tests might be used quantitatively with purified preparations, the antisera used were from animals injected with concentrates. Apparently agglutination of virus particles by convalescent sera has not been reported. Infection of chick embryos has been reported with as few as 10 particles (62). These repeated studies have related viral activity to particles about 100  $m\mu$  in size present in the allantoic fluid.

The filaments of influenza virus, seen more commonly in recently isolated strains, i.e. strains which have been through fewer passages in the embryo, are more difficult to relate to infectiousness. They may even predominate in the allantoic fluid (63). Their association with infection of the allantoic sac and of cells of this area grown in tissue culture (14) is well-established (14, 64, 65). Because of previous difficulty in separating the filaments from the spherical particles, even by centrifuging allantoic fluid, it has been difficult to obtain direct evidence concerning their viral nature. They have been adsorbed on red cells (63, 66) and they may be agglutinated by antisera. No reports on agglutination by sera from convalescent animals have been published. Recently Donald & Isaacs (66) have taken advantage of the spray type of counting procedure to determine the number of filaments and spherical particles present in different preparations and have estimated that filamentous strains of virus agglutinated standard red cell preparation when  $10^{6.6}$  particles were present; for spherical forms  $10^{7.2}$  particles were necessary. Filaments were partially separated by filtration through a collodion membrane. When these were broken down by ultrasonic bombardment, there was a four-fold increase in the hemagglutination titer. There was no demonstrated increase in the infectious titer. The authors conclude that the filament is 7 or 8 times as efficient as a sphere in producing agglutination in the pattern test.

Thus there is direct evidence relating the filaments to the infectious particle, but no data which indicate whether one filament contains more than one infectious particle within it, although it would seem that multiples of hemagglutinating activity were present. Examination of the infected tissue itself either in sections (64, 65, 67) or in tissue culture (14, 65) shows that filaments may frequently consist of chains of spherical particles, which are presumably breaking out of these filaments. It seems likely that these filaments arise from the surface of the cell, either by altering the normal microvilli, or by modifying the cell's tendency to form such microvilli. Thus the filament of influenza virus might be considered either as an abnormal extrusion of the cell in which the virus might continue to increase in amount (55b), or it may be considered as an elongate virus particle which has failed to divide into its component units—much like an abnormally elongated bac-

terium growing under the influence of sublethal amounts of penicillin. Present methodology does not allow a judgment and indeed the difference may be more a point of view, rather than a fundamental one.

*Incomplete Virus.*—Under certain condition admittedly unfavorable to maximum growth of this virus, i.e., following intracerebral inoculation in mice or the inoculation of large amounts of virus into the allantoic sac "incomplete" virus particles may be produced. These are particles containing the hemagglutinating activity but lacking infectiousness. The appearance of these following purification has been studied by Werner & Schlesinger (68) who showed that they might be identified by adsorption on to red cell ghosts. They do not have the uniform compactness of the "complete" particle, and seem to pancake on drying. The majority appeared "bag-like and had a rough granular surface and reduced uneven density." In general they are larger, with 37 percent of the particles with a diameter in excess of 170 m $\mu$ . This may be related to their flat shape when dried.

*Newcastle disease virus.*—The virus of Newcastle disease of chickens is recognized as a member of the group, which includes influenza, mumps, and fowl plague. It was early shown to have a peculiar appearance when prepared by drying from salt solutions on a collodion screen (69, 70 to 73). Shortly afterwards it was pointed out that the particles as such did not have a sperm-shaped morphology when prepared either from allantoic fluid or from water suspension (73, 74) but were essentially spherical. It was unfortunate that the adjective "filamentous" was used at that time, for although the distorted form which is seen in the dried preparations often is thread-like, this has no known relation to the "filaments" of influenza. In this discussion we will refer to the peculiar appearance of Newcastle virus, and also of avian erythromyeloblastic leukosis as pleomorphic, and will reserve the word filament for those long threads of fairly uniform diameter, which are of the same thickness as the individual particles.

Newcastle virus has a peculiar tendency to change its shape in salt solutions of different concentrations. This may be demonstrated by fixing the virus with osmium tetroxide while it is suspended in 0.15M saline and then transferring it back to water from which it is dried on the collodion screen (75). If the virus is fixed in normal physiological saline and subsequent preparations made, no change of shape appears (75). Similar spherical particles were obtained by Sharp *et al.* when the virus was treated with formalin before drying. The effect of the increasing salt concentration in drying may be avoided by centrifuging the virus on to an agar block as advocated by Sharp (76) or by fixation before drying.

This characteristic distortion is of considerable help in identifying the agent and is similar in many respects to the pleomorphic appearance of several members of the pleuropneumonia group of agents. Two recent electron microscope studies of these latter agents show that the great majority of these bacteria-like agents appear as flattened discs when dried from the



colony after osmic acid fixation. Thus their pleomorphic character is also inhibited by adequate fixation (77, 78).

The spherical form is apparently the form to be expected in physiological solutions. These have been found in allantoic fluid (6, 74, 76) and in tissue culture (79) and have been associated with the infected tissue (79). It was early shown by centrifugation that particles of about 100  $m\mu$  were associated with both infectivity and hemagglutination (6, 69, 70). The particles may be adsorbed on to red blood cells and studied there (80). Agglutination of the particles by convalescent chicken sera has been reported. Infection of the chick embryo (6, 69) may be produced with about five particles (6), which at the time of study was within the limits of the methodology.

Little attention has been paid to possible internal structure in these particles. Yet the very peculiar shape assumed by the virus when dried would suggest some basic structural organization. Dense central portions are seen in the virus adsorbed on red cells (80) and were particularly noted in later preparations of allantoic fluid virus in which the virus was fixed while in the allantoic fluid (79).

The appearance of the virus has been studied both within the cell in sections of the chorioallantoic membrane (79) and in tissue culture (79). Sharply defined morphological units have not been found, and emphasis has been placed on the relation of the virus to the surface activity of the cell. It should be emphasized, however, that this relationship is apparent with a strain which does not spread rapidly through the tissues. The virulent strain of virus, however, destroys cells, produces both cytoplasmic and nuclear changes, and is thought to be present in the cytoplasm. Actual description of its morphology within the cell may now be possible with the higher resolution available on extremely thin sections.

As with influenza virus, it is at present not possible to separate host from virus in the surface area of the infected cells. Spherical particles are found abundantly scattered among the altered and fungus-like mass of microvilli and ballooned processes (79). The particles in tissue culture preparations, except for the size difference, are very similar in appearance to the forms of pleuropneumonia studied by Turner in a direct examination of living material by dark field microscopy (81).

Recent reports from Japan (82) indicate that a virus belonging to the mumps-NDV-influenza group may be recovered from mouse populations by serial passage of lungs. Electron microscopy (83) of the allantoic fluid virus showed many particles averaging 160-180  $m\mu$ , which were "irregularly shaped, sometimes elongated like a finger, or sometimes with a tail-like appendix like a comma, in Ringer or saline solution, while, if it was suspended in distilled water or fixed in formaldehyde solution it appeared like a flattened cube." The authors point to the similarity in behavior and morphology to Newcastle virus. Although this virus apparently produces lesions in mice and differs somewhat antigenically from some strains of Newcastle virus, it has

so many similarities to this virus, that further direct comparisons to a variety of strains is necessary before we can state whether the virus is an antigenic variant of NDV or not. It is clear that the evidence relating the morphological unit to the infectious particle is good.

*Mumps.*—The first good electron micrographs of mumps virus were made by Weil *et al.* on preparations obtained from the allantoic fluid of infected chick embryos. The particles dried from Ringer's solution had "an ameboid appearance with single and multiple pseudopodia-like processes extending from the peripheries of the particles" (84). In the light of present knowledge concerning distortion produced by drying from saline, it is likely that this does not represent their true shape. Indeed the authors state that "a somewhat better contrast and demonstration of the particles, as well as far greater uniformity in shape, can be obtained with preparations treated with formalin."

The presence of these fairly characteristic particles in the allantoic fluid of infected embryos and the association of their presence with both infectivity and hemagglutination as brought out by differential centrifugation are valid reasons for believing that the particles of  $171 \pm 31.3 \text{ m}\mu$  (formalinized) are the virus. Such particles may be found adsorbed on laked red cells (59). These morphological findings have been confirmed with three other strains of mumps virus, and it was further shown that although some normal allantoic fluid pools contain similar particles, these were not absorbed on to red cells.

As no studies of the genesis of the virus particles from the chorioallantoic membrane have been reported, it is not possible to relate the formation of the particle to cellular events.

*Fowl Plague.*—This virus is a well-recognized member of the group, but has been differentiated from Newcastle disease on the basis of size, antigenicity, and behavior. Purified preparations were studied in the electron microscope in 1950 by Schafer & Schramm (85) who showed that infectivity and hemagglutinating activity were closely correlated with particles of about  $100 \text{ m}\mu$ . When purified material was dried from water, flat discs of  $117 \pm 4 \text{ m}\mu$  with poorly outlined edges were found. When dried from saline, the particles were  $81 \pm 2 \text{ m}\mu$ , and predominantly spherical or oval. An occasional particle was extended but no forms similar to Newcastle virus were seen. The salt solution tended to make the particle more compact. This work was carried further in the same laboratory (86) and, by fractional centrifugation and electrophoresis, a more purified preparation was obtained. With osmium fixation the round forms were preserved and the diameter was estimated at  $70 \text{ m}\mu$ .

The development by Dawson & Elford (59) of the technique of adsorbing viruses from the allantoic fluid brought to light filaments as long as  $6\mu$  in the infected fluid of fowl plague. These were adsorbed on to the red cells and, like the filaments of influenza, had the same diameter as the spherical



particles. A tissue culture study using chick amnion and pectoral muscle, and infected with fowl plague has further linked the filaments with the virus (87). Flewett & Challice found a profusion of filaments appearing from cells 36 to 48 hr. after infection. A few of these showed segmentation into spherical bodies and shorter filaments.

Further study by the workers at Tubingen [Hotz & Schafer (88)], in which direct examination of thin sections of chick chorioallantoic membranes infected with large amounts of virus was made, has emphasized again the importance of surface growth, and the relation of filaments thereto. The effect of the virus was followed within the first few hours after infection. At 4 to 5 hr. there first appeared a mass of protrusions in the form of vesicles and filaments. At 6 hr. a great number of particles was seen and these were similar to virus particles in appearance. The latter seem to arise through division of the filaments or at the free end. They were not seen inside the cells.

As with influenza viruses, there is the question of "incomplete" forms. Schafer and co-workers (89) have prepared virus of a low infectivity and hemagglutination ratio from homogenized chorioallantoic membrane. Sedimentation of material obtained by adsorption and elution from red cells showed heterogeneity of size. Electron microscopy indicated particles ranging in size from 50  $m\mu$  to 550  $m\mu$  but there were mostly collapsed bags—appearing flat on the screen. A variety of treatments of "normal" virus failed to produce particles similar in appearance.

#### TUMOR VIRUSES

Several agents are now well-recognized as capable of causing either benign or malignant hyperplasias. The best known agent is the Rous sarcoma virus. Little adequate electron microscopy has been published concerning this agent. The difficulties of purification have been so great that this material has apparently not been satisfactory for such examinations. Thus available information is derived from studies of cells in tissue culture. Claude *et al.* (90) showed that numerous characteristic scattered individual particles of about 67 to 80  $m\mu$  and clumps of particles may occur in tumor cells. They were apparently not found in normal cells. Recently Bernhard *et al.* (91) have confirmed this and have emphasized the need for studying degenerating cells. The particles appear characteristic, numerous, and fairly uniform in size, about 60 to 70  $m\mu$ . Again such forms were not found in control preparations. Similar particles have been found in the sections of tumors (92).

However, the problem is complicated by the finding by Bang that about 15 per cent of the "normal" chick embryo cultures prepared in his laboratory (55) produce similar degenerating cells containing particles identical in appearance. Through the courtesy of Dr. W. Bernhard, direct comparisons of his "virus" particles and our "degenerating" cell particles were made. They could not be differentiated on size, shape, or arrangement. However, the

presence of these particles in the normal cultures is not invariable, and it is known that the flock of chickens which furnished the embryos used in these experiments had antibodies to the Rous virus and was thus presumably infected with this agent. Since few flocks of chickens are known to be free of the agent, the choice of controls becomes extremely difficult.

*Mammary Tumor Inciter.*—A number of pictures of material obtained by centrifuging and purifying milk from mice known to carry this agent have been published (93). However, at no time have these particles been shown to have a characteristic appearance, nor has it been possible to obtain adequate controls of concentrated material from normal mice which show no similar particles. Tissue culture preparations of mammary carcinoma tissue have shown cells with clumps of virus-like particles (94). These particles, which were assumed to be the milk agent, had dense central portions of about 75  $m\mu$  in diameter while the periphery exhibited greater variation, averaging about 130  $m\mu$ . Particles were found in 3 of 6 preparations from spontaneous and transplanted tumors. Recent preliminary reports (95 to 98) on sections of the mammary tumors indicate the frequent occurrence of similar particles. These may be seen extended from the cell in microvilli as well as deep in the cell. They have not been found in normal mammary glands. It has not been established that they are characteristic of mammary tumors initiated by the milk agent alone.

*Shope Papilloma.*—This virus, causing an infection in rabbits, was studied early in purified preparations by Sharp, Taylor & Beard (99) and found to be a spherical particle of about 50  $m\mu$ . Recently the application of uranyl nitrate as an electron stain has brought out several small dense regions of about 8  $m\mu$  in size (100), and careful measurements of the unshadowed particle show it to be  $46.9 \pm 5$   $m\mu$  in diameter. When the height was estimated from shadowed specimens, it was concluded that the particle was flattened (27  $m\mu$ ), presumably by drying, and that a corrected figure for the particle before drying would be 40  $m\mu$ .

*Human Papilloma (Warts).*—Although the virus etiology of this skin condition rests on relatively few transmission experiments in man, the electron microscope studies of extracts of tissue and of thin sections of the papillomas have revealed such characteristic particles that they should be included in this review. Large numbers of particles about 52  $m\mu$  in size were first found in water extracts of certain plantar and common warts (101). The lesions which yielded the particles differed from other ones in this clinical complex in that intranuclear inclusions and cytoplasmic masses were commonly present in these warts which yielded the "crystalline" array of particles (102). Control preparations consisting of other kinds of warts, of normal skin, and of molluscum contagiosum, showed none of these particles. Thin sections of these lesions have shown that the particles are packed together in the nuclei of the cells in the neoplastic epidermis (103). In the cells of the stratum corneum where the nucleus is not recognizable, the parti-

cles occupy all of the cell except for a narrow peripheral border. Presumably the close packing is responsible (103) for the crystalline like array.

*Avian Erythromyeloblastic Leukosis.*—One member of the general leukosis complex (104) of chickens (avian leucosis), has been given the above descriptive label by a group of workers (76, 105) at Duke University. Purification of the virus from the plasma of infected birds by differential centrifugation revealed the presence of many particles of about 120  $\mu$  in diameter. These particles had the same characteristic distortion on drying from saline (76) which had been found to be true of Newcastle virus when dried from saline (75). The authors point out the similarity of this effect and were able to avoid the distortion by centrifuging the virus on to agar surfaces which adsorbed the salt.

The evidence for the viral nature of the particles frequently found in the serum is more complete than that for several other tumor viruses. Not only has the infectious activity been associated with the particle by differential centrifugation and electrophoresis, but certain enzyme activity (ATPase) has been found to be closely correlated with the infectious activity of the suspensions of virus (76a). If, however, the virus emerges from cells in a manner similar to Newcastle virus (79) it would be hard to distinguish host activity from virus activity. A few particles are found in normal birds (106). As yet no data on the origin of the infectious particles are available.

Similar particles were later found in the plasma of chickens suffering from lymphomatosis (106), which is another member of this group of agents. Few or no such particles were found in normal plasma. These findings have been confirmed (107).

#### ENCEPHALITIDES

*Equine Encephalomyelitis.*—Although there are several strains of agents, differing antigenically and in host range, there has been little done with this group of arthropod borne encephalitides except eastern and western equine encephalomyelitis. Electron microscope study of purified preparations obtained from infected chick embryos by ultracentrifugal procedures showed a group of particles of about 47.5  $\mu$  for the eastern strain and 53.1  $\mu$  for the western strain (108, 109). Studies of tissue cultures of chick embryos infected with the eastern strain have shown a profusion of particles emerging from the destroyed cell (110, 111). Similar particles were found partially destroying the susceptible cells in rat fibroblast cultures (112). Although some variation in size and density has been recorded (108, 112), there has been no study of the internal structure of the virus.

*Encephalomyocarditis.*—This agent has been purified from mouse brain by protamine precipitation and cleaned up for electron microscopy by digestion with trypsin. Relatively few pictures of this purified material have been published (114).

## POLIOMYELITIS-LIKE VIRUSES

*Coxsackie*.—A host of different strains or agents are now included under this label. There has been no general attempt to study them with the electron microscope. Two members of the A group of these viruses have been purified from infected chick embryo amniotic fluid and from infected mouse muscle and studied in shadowed preparations. Under these conditions their size was about  $37\text{ m}\mu$  (113). Smaller particles were found in the preparations but these also were obtained from normal tissues (113). When normal particles are present in large number and have the same size in the tissue, it has not been possible to differentiate the virus from them (114).

*Poliomyelitis*.—The viruses causing human poliomyelitis are now recognized as belonging to three antigenic types. A recent review (115) of the evidence for the identification of these agents in the electron microscope pointed out that the agent until recently had not been identified. The recent concentrated effort to produce a killed vaccine resulted in several preparations of virus which were suitable for electron microscopy. Bachrach & Schwert (124) studied purified preparations of cotton rat brain infected with virus, and, by means of particle counts, showed that there was a correlation between particles of about  $28\text{ m}\mu$  in diameter and the infectivity of their material as it was purified. They emphasize the uniformity of their material and believed that normal particles could be differentiated on the basis of size.

The introduction of tissue culture techniques has furnished new material for electron microscopy. Several reports (115, 116, 117) agree that the sizes of the viruses are about 28 to  $30\text{ m}\mu$  but differ as to the degree of uniformity of size.

In our listing of the evidence relating these particles to virus activity, we have listed only one reference indicating a characteristic appearance. This reference is tentatively put in this column because the clumps of particles seem suggestive. However, none of the published reports show particles which on direct examination could be identified as characteristic of viruses, either individually or as a group. No reports showing the relationship of the agent to the infected cell have been published and no attempts to visualize the interior of the particle have been published. The report of virus within "neurotubules" of the axons of the nerve (118) has to be considered in the light of the likelihood that the "neurotubules" are really collagen fibers around the axon (120).

*Theiler's Virus*.—This agent is somewhat related to the poliomyelitis group and has been concentrated and purified from mouse brain (119). Characteristic clumps of particles of  $28\text{ m}\mu$  are illustrated. A few irregular individual particles were found in normal mouse brain.

## SUMMARY

We have had two general purposes in this review. The one was to list and evaluate the evidence relating a given particle to a given virus. The

other was to assess our knowledge concerning the actual morphology and structure of the particle.

The table listing the references to available data gives some idea of the mass of literature on the first point. It is clear not only from the number of criteria fulfilled for some of the viruses, but also from the careful and repeated nature of some of the studies, that several viruses can be rather definitely identified as having a certain size and shape as seen in the electron microscope.

In the case of the pox group of viruses, enzymatic analysis of the individual virus has shown that the central dense portion has some properties of nucleic acid, and that the external membrane which is left behind after combined peptic and deoxyribonuclease digestion is similar to the bacterial cell wall. This suggests that the pox group of viruses is related to bacteria.

Pleomorphism under differing salt concentrations, which leads to distortion during drying, is apparent in Newcastle virus and in the larger pleuropneumonia organisms, and results in developmental forms which differ only in size. The pleuropneumonia agents and the Mumps-Newcastle-Influenza virus group (myxovirus) are common residents of the mucous membranes, particularly of the upper respiratory tract. Thus a similarity in their morphology and development suggests that these two groups of agents may be related.

Our terminology has certain contradictions. When considering the pox viruses and the psittacoid group, we continually referred to elementary bodies but, when referring to encephalomyelitis and poliomyelitis, to virus particles. This dichotomy arises chiefly from the different groups of workers who have studied the large and small viruses. We would suggest that when the evidence is strong that a given particle is the virus, that we simply speak of it as the virus. When the evidence is not strong but suggestive, we may call it virus-like particle. This does not preclude some contribution on the part of the host cell to the given virus, and this will have to be determined for each virus.

Several viruses smaller in size than the pox group have varying amounts of dense material within the central portion. A beginning of the determination of the structural makeup of these smaller viruses is now possible. Most studies of the smaller viruses have emphasized shadowed preparations which prevent any internal differentiation.

There is as yet no adequate morphological study of how viruses of any size multiply or reproduce. Such studies must be carried out in some sort of time sequence, and only suggestions can be derived from the study of one set of sections. However, the tremendous detail now available in very thin sections, such as those of Morgan and co-workers, demonstrate that this is now possible. Double forms and chains of particles have been recorded in several infections, suggesting that some of the processes may be like those in higher forms, but these will have to be obtained in strict sequence—either

in tissue culture preparations or in infected tissue cut in thin sections. The latter is more subject to distortion during the process of embedding, and thus both whole mounts and thin sections need to be studied. It is unlikely that continued studies of purified and thus standardized preparations alone will give us the morphological data essential to an understanding of the parasitic relationship of virus and host cell.

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## GENETICS OF MICROORGANISMS<sup>1,2</sup>

By M. R. ZELLE

*Laboratory of Bacteriology, Cornell University, Ithaca, N. Y.*

### INTRODUCTION

In the present state of rapid development of knowledge of the genetics of microorganisms, an attempt to prepare a review is a formidable and, in some respects, almost hopeless task. The already broad horizons are so rapidly being extended that it would be presumptuous for the present writer, at least, to pretend to an authoritative knowledge of all of the various phases. The growth of the field is evidenced by a parallel increase in the literature to a volume which precludes any possibility of achieving both criticalness and inclusiveness within the limitations imposed by the available space.

This situation has been recognized by the editorial committee of this volume and, in the future, the present series will be replaced by a number of reviews concerned with limited areas of the subject. In the meantime, it seemed that the present review might be of greatest service if it were made sufficiently inclusive to provide a useful index to the literature of the past year. The broad outline followed in last year's review (392) has been retained since it serves this purpose well.

Although a conscientious effort at complete coverage has been made, it is inevitable that unfortunate and unavoidable omissions will have occurred. The 475 references included were selected from a total of more than 800 and the writer can only ask for the reader's indulgence if his choices do not always appear to have been wise. In accordance with the expressed policy of the journal, no references have been made to material published in the *Microbial Genetics Bulletin* without the specific permission of the authors. The field of bacterial cytology has been considered as beyond the scope of this review.

Several books which will be valuable to students of microbial genetics were published during the year. Haldane's book (168), though ostensibly written for the biochemist, utilizes much information derived from microbial genetics and is a stimulating essay for all geneticists. Beale (34) has summarized the genetics of *Paramecium aurelia* in a lucid manner which is informative and accessible to the uninitiated and, at the same time, a thought-provoking consideration of the implications of cytoplasmic inheritance in *Paramecia* for the problem of cellular differentiation. A valuable symposium concerned with sexual reproduction in a number of microbial

<sup>1</sup> The survey of literature pertaining to this review extends through December, 1954.

<sup>2</sup> The following abbreviations have been used in this chapter: DNA (deoxyribonucleic acid); RNA (ribonucleic acid).

forms has been published (443). In addition, two volumes, concerned at least in part with problems of microbial genetics, may be mentioned (51, 173).

## PHENOGENETICS

### GENETIC ANALYSIS OF INTERMEDIATE METABOLISM

Certain phases of the genetic analysis of intermediate metabolism have been reviewed by Horowitz & Owen (196).

*Amino acids.*—Kalan & Ceithaml (217) have isolated four different groups of methionine requiring mutants in *Escherichia coli*. The growth responses of the four groups are consistent with the hypothesis that a four-carbon,  $\alpha$  amino acid is a precursor of methionine. Friedman (143) showed that  $\alpha,\gamma$  diaminobutyric acid could replace the requirement of an *E. coli* auxotroph requiring either  $\alpha$  amino butyric acid, homocysteine, or methionine whereas  $\beta,\gamma$  diaminobutyric acid could not. A methionine-requiring mutant of *Aerobacter aerogenes* (379) apparently is unable to synthesize thiomethyladenosine which is required for methionine synthesis. It is postulated that methionine is synthesized by a transthiomethylation of some four-carbon chain compound, perhaps  $\alpha$  aminobutyric acid. Isotopic competition experiments in *E. coli* confirm biosynthetic pathways postulated for a number of amino acids (2).

Vogel & Bonner (425) conclude that the glutamate-proline-ornithine interrelation in *Neurospora* is very similar to that in mammals but different from that found in *E. coli* (424). They suggest, on the basis of the apparent non-availability of endogenous ornithine for proline synthesis, for example, that *Neurospora* may be so organized that enzyme systems involved in ornithine and proline synthesis are physically separated with a consequent "channeling" of metabolites.

Based on studies of a number of *E. coli* mutants Cohen & Hirsch and their collaborators (80, 81, 298) have elaborated the synthetic pathway by which threonine is synthesized from aspartic acid. Conversion of homoserine to threonine has been observed in extracts of certain mutants and, in general, those mutants blocked between homoserine and threonine do not possess the enzyme system although there are exceptions to this rule. These same workers have also demonstrated an enzyme system forming homoserine from aspartate (190). Tracer studies (151) have confirmed homoserine as a precursor of threonine in *Neurospora* but not of methionine as had been concluded earlier. Threonine was also shown to be a precursor of isoleucine.

Tatum *et al.* (413) conclude that the *arom* mutant of *Neurospora*, which requires for growth para-aminobenzoic acid, tryptophan, phenylalanine, and tyrosine or their common precursor, shikimic acid, has a genetic block preventing the formation of shikimic acid from dehydroshikimic acid. Instead of being accumulated, the dehydroshikimic acid appears to be converted to protocatechuic acid. Labelling studies in *E. coli* (395) indicate that, in the conversion of shikimate to tyrosine, the carboxyl of shikimate is eliminated

and the side chain enters the same position. The side chain may be derived from a three-carbon compound originating in glycolysis. Davis (91) summarizes the knowledge of the synthesis of the benzene nucleus. Tatum & Shemin (414) studied the condensation of indole and serine to form tryptophan in *Neurospora* with multiply-labelled serine. Their results suggest that the condensation involves the intramolecular dehydration of serine. A detailed mechanism for this reaction has been elaborated (171). Hogness & Mitchell (191) showed that in *Neurospora* the concentration of tryptophan desmolase, the enzyme catalyzing the indole-serine condensation, can be influenced by genes other than those causing a tryptophan requirement. Restoration of apparently the same enzyme can occur either through back mutation or by the action of an independent suppressor gene.

Weijer (440) reviews much of the literature concerned with the *td* (tryptophan desmolyase) locus in *Neurospora* in a report of his studies of recombination within the *td* region. He did observe tryptophan-independent isolates among the progeny of some crosses between 24 different *td* mutants but their origin is still not clearly determined. Weijer also puts forth the interesting hypothesis that temperature-sensitive mutants may be due to a change in properties of the matrix membrane of the chromosome. Tryptophan-requiring mutants of *E. coli* were found by Gots *et al.* (160) to fall into five distinct phenotypes as determined by their response to indole and anthranilic acid and by the substances accumulated. Tryptophan-requiring T1 phage-resistant mutants differ from the other types in a number of respects.

Studies of an asparagine-requiring mutant of *Neurospora* (412) have not yet disclosed the synthetic pathway of asparagine beyond the conclusion that asparaginase, present in both the mutant and wild type, is probably not involved. Barratt & Ogata (29) investigated a mutant requiring for growth any one of a number of structurally unrelated amino acids. They postulate that the active compounds reverse the action of an inhibitor accumulated in the mutant.

Wild type strains and mutants of *E. coli* and *Neurospora* with a double requirement for isoleucine and valine have been assayed for their content of dihydroxy acid dehydrase, an enzyme which converts the dihydroxy acid precursors to the corresponding keto acid precursors of each of the amino acids (286). The *E. coli* mutant had no detectable activity; the *Neurospora* mutant had less than 10 per cent of the activity of the wild type. Similarly, *Neurospora* strains carrying the mutation *am* require  $\alpha$ -amino nitrogen for growth (127) and possess no detectable glutamic dehydrogenase activity although as little as 0.2 per cent of the normal wild type activity could have been detected. Adams (4) has shown that the presence or absence of an enzyme converting histidinol to histidine in a given *E. coli* histidine-requiring mutant is correlated with the ability of the mutant to utilize histidinol for its histidine requirement.

A *Neurospora* mutant inhibited by threonine (428) accumulates  $\alpha$ -keto-

$\beta$ -ethylbutyric acid, a derivative of threonine and a precursor of isoleucine. The mechanism appears to be a competitive interference with the metabolism of homocysteine (103). Studies of the effect of temperature suggest that growth inhibition by competitive mechanisms may be one basis for temperature sensitive mutants (103). A histidine-inhibited mutant of *Neurospora* has been isolated (339), and preliminary evidence indicates that histidine may interfere with transamination.

Differences in the free intracellular amino acids of *Neurospora* mutants have been observed (145). A proline-requiring mutant has been isolated in *Shigella dysenteriae* (216) and several amino acid-requiring as well as other types of auxotrophic mutants have been isolated from a rather atypical strain of *Mycobacterium tuberculosis* following ultraviolet radiation (219).

*Purines and pyrimidines.*—Nelson & Shapiro (290) have isolated four distinct types of pyrimidine auxotrophs in *Aerobacter aerogenes*. They postulate a synthetic sequence leading from oxalacetate through aspartate, ureidosuccinate, and orotate to uracil. The formation of orotic acid in pyrimidine-requiring mutants of *A. aerogenes* was shown to be inhibited by uracil, presumably by inhibition of an enzyme involved in the conversion of an unknown precursor to orotic acid (64).

A purine-requiring mutant of *E. coli* which accumulates 4-amino-5-imidazole carboxamide has been used in studies of the nitrogen, carbon, and energy requirements for the synthesis of this compound (161). A potassium requirement for purine synthesis was also disclosed by studies of this mutant (144).

Love & Gots (253) have studied another *E. coli* purineless mutant which accumulates a compound believed to be an aminoimidazole ribotide (or riboside) which functions as a precursor to the ribose derivative of 4-amino-5-imidazolecarboxamide. A tentative pathway of purine synthesis in *A. aerogenes* is suggested by Brooke & Magasanik (63) from studies of two purine auxotrophs, one of which accumulates xanthosine, the other being unable to synthesize the ribotide of 4-amino-5-imidazolecarboxamide. Aaronson & Nathan (1) suggest that amidine (4-amino-5-imidazolecarboxamidine) may enter into purine synthesis more directly than 4-amino-5-imidazolecarboxamide. This suggestion rests largely upon the ability of amidine to support growth of *Crithidia fasciculata*, a purine-requiring trypanosome parasite of mosquitoes.

*Vitamins.*—The synthetic chain leading from tryptophan to nicotinic acid in mammals and *Neurospora* has been elaborated in recent years. A biotin antimetabolite inhibits the utilization of tryptophan by a niacin-requiring *Neurospora* mutant (403) and biotin was shown to function in the formation of formylkynurenine from tryptophan. Further support for the view that formylkynurenine is an intermediate in niacin synthesis in *Neurospora* was supplied by the isolation and partial purification of an enzyme which converts this compound to kynurenine (212). Yanofsky (465) utilized

a series of tryptophan and niacin requiring mutants of *E. coli* and *Bacillus subtilis* to show the absence of a tryptophan-niacin relationship in these species.

The ability of a number of compounds implicated in methionine synthesis to support growth of a series of mutants of *E. coli* with vitamin B<sub>12</sub> requirements ranging from 0.018 to 0.12 µg. per ml. has been tested (105). Although certain intermediates could support growth of mutants with relatively low requirements, only B<sub>12</sub> and methionine could support growth of the mutants with the highest requirements. The inability of methionine to support continuous subculture of such mutants indicates that B<sub>12</sub> is also required in *E. coli* for reactions other than methionine synthesis.

A mutant yeast strain which excretes riboflavin has been employed (156) in tests of the effect of various purines, pyrimidines, and amino acids on riboflavin synthesis.

An unusual mechanism has been found to be responsible for a thiamine requirement in a mutant strain of *Saccharomyces cerevisiae* (415). The mutant contains a phosphatase which hydrolyzes cocarboxylase but which is inhibited by thiamine. The wild type strain possess only very slight phosphatase activity.

Reduced permeability is postulated as a major factor in the resistance of a *Streptococcus faecalis* mutant tolerating large amounts of folic acid antagonists (294).

Garber (150) has continued his studies of the influence of auxotrophic mutations on the host-parasite relationship in plants. In similar studies on animal pathogens, a strain of *S. typhosa* (128) avirulent for mice was found to have an additional purine requirement. Mouse peritoneal fluid could not supply sufficient purines for growth and injection of xanthine along with the bacteria significantly increased virulence. Reversion to purine independence was accompanied by increased virulence.

*Pigment biosynthesis.*—A beginning in the genetical analysis of carotenoid biosynthesis in microorganisms has been made. A series of mutants of *Corynebacterium michiganense* has been examined for their content of various carotenoids and for the influence of thiamine on pigment synthesis (369). Claes (77) has isolated four mutants of *Chlorella vulgaris* which show blocks in the synthetic pathways of the carotenoids. Some of the accumulated carotenoids have been identified. A short exposure to light in the presence of oxygen was shown to be necessary to initiate carotenoid synthesis in *Neurospora* (467). Independent synthesis of a number of carotenoid pigments was observed after exposure to light.

Flavonoid biosynthesis has been studied in a series of noncopulating mutants of *Chlamydomonas eugametos* which are unable to synthesize the hormone quercetin (46).

Rizki (352) has discovered a diffusible chromogenic inductor in *Serratia marcescens* which is able to induce formation of the wild type red pigment



in color mutants. The interactions of twelve strains have been determined and spectrophotometric evidence (353) showed that the induced pigment is similar to that produced in the wild type.

*Cytochrome system.*—Laskowski (234) has shown that 2,3,5 triphenyl-tetrazolium chloride induces the "petite" colony mutation in yeast under conditions where selection of a rare mutant is excluded. The induced mutants are physiologically and morphologically similar to the spontaneously occurring respiratory mutants. The action spectra for ultraviolet inactivation of both normal and cytochrome-deficient haploid yeast was found to be of the nucleic acid type by Raut (343, 344) who observed a high proportion of "petites" among the colonies from survivors of normal cultures. This cytoplasmically determined cytochrome deficiency also exhibits a maximum rate of induction at 2600 Å. These observations lend support to the view that the "petites" result from the loss of self-reproducing, non-genic cytoplasmic entities that contain nucleic acid.

Bautz & Marquardt (33) have observed that, whereas normal *Rhodotorula rubrum* cells have about 12 Nadi-positive mitochondria, approximately 2 per cent of the cells are Nadi-negative and possess about nine mitochondria per cell as shown by the Altmann staining method. No intermediate cell types were observed. Nadi-negative cells could be selected by incubation in the presence of the Nadi reagent and x-rays significantly increased the frequency of Nadi-negative cells (32). Nadi-negative cells were smaller, grew more slowly, seemed not to possess a normal cytochrome system, and invariably seemed to revert to normal after continued growth. Respiratory mutants of *Saccharomyces* were shown to be unable to oxidize the Nadi-reagent (174).

Ogur (302) observed that the rate of respiration and aerobic fermentation of glucose in a polyploid series of yeasts was almost perfectly correlated with the degree of ploidy, suggesting that the enzyme content is quantitatively dependent on the quantitative genetic complement.

The cytochrome content and respiratory activities of some cytoplasmically and genetically determined respiration mutants of *Neurospora* has been re-examined (418). Certain yeast strains and their "petite" mutants were shown to contain oxyhemoglobin (220). Beljanski (38) observed a decreased iron content and the absence of certain cytochrome bands in a streptomycin-resistant mutant of *E. coli*.

*Carbohydrate metabolism.*—Gilliland (154) isolated a wild yeast, *Saccharomyces diastaticus*, which was found to differ genetically from *S. cerevisiae* at a number of loci. Allelism tests have been made among the multiple genes controlling maltase formation in this new yeast and in a number of other species (153, 454).

The long-term adaptation of yeast to galactose has been discussed in a number of papers. Further evidence strongly supporting the mutation selection interpretation has been presented (113, 321) although the possibility of cytoplasmic interactions as a modifying agency (321) or as a major factor



(114) has been recognized. The finding of Douglas & Condie (104) that two genes ( $G_1$  and  $G_2$ ) are involved in the genetic control of galactose utilization in *S. cerevisiae* may remove some of the confusion which has existed. The dominant  $G_1$  appears to control the formation of an enzyme converting galactose to glucose-6- $PO_4$  whereas  $G_2$  is believed to be involved in the transport of galactose into the cell. Cells with the  $g_1 G_2$  genotype are unable to utilize galactose whereas  $G_1 g_2$  cells are slow fermenters.  $G_1 g_2$  or  $g_2 G_1$  clones can undergo long-term adaptation to rapid fermenters by mutation at only one locus although the  $G_2 g_1$  clones are slow fermenters and produce gas before an appreciable mutant population has developed. Clones of the  $g_1 g_2$  genotype are unable to grow at the expense of galactose and would only very rarely produce a rapid fermenter since coincident mutations would be required.

Strauss (401, 402) has shown that the formation and accumulation of pyruvate in acetate-requiring mutants of *Neurospora* is inhibited by the presence of acetate. Glucose and sucrose inhibit the acetate mutants, probably through the formation of acetaldehyde or a derivative formed from it. Two different suppressor mutations relieve the inhibition by lowering the activity of pyruvic carboxylase, which results in a decrease in acetaldehyde and ethanol formation and permits some growth in the absence of added acetate.

A mutant strain of *Bacillus subtilis* which does not oxidize glycerol through the tricarboxylic acid cycle has been shown to lack a triosekinase present in the wild type (448). A mutation affecting glucose and lactic acid metabolism in the pneumococcus has been shown to be transmissible by transforming extracts in a manner analogous to capsular characteristics (120).

From *Chlamydomonas dysosmos*, a facultative photoautotroph, Lewin (248) isolated a mutant which was no longer able to utilize acetate for heterotrophic growth in the dark. The mutant behaved like an obligate photoautotroph and, although it oxidized all added acetate to  $CO_2$ , it was unable to utilize the energy for growth and synthesis.

Weed & Longfellow (437) have isolated a small colony variant of *E. coli* after growth in the presence of copper ions which exhibits marked differences from the parental strain in carbohydrate and nucleic acid metabolism.

#### MORPHOLOGICAL AND SEROLOGICAL VARIATION

An extensive survey by a number of authors of morphological and other variations in relation to speciation in the asexual fungi (273) draws attention to some of the problems of microbial taxonomy. The S/R and O/o variations in the gram-negative intestinal bacteria have been reviewed by Kröger (226).

*Antigenic variation.*—Transduction analysis has shown the two alternative phases of *Salmonella* flagellar antigens to be controlled by two distinct loci,  $H_1$  and  $H_2$ , corresponding to the two series of antigens (240). The correlation observed between the antigenic phase of the donor cells and the

transductive competence of their phage lysates prompted Lederberg (240) to suggest that the mechanism of genetic differentiation of the phases may be based on the states of the  $H_1$  and  $H_2$  loci themselves. Bruner (65) reports a second case of induced variation in *Salmonella* somatic antigens within an antigenic group.

Serial transfer in broth containing heat killed Vi cells, saline extracts of their acetone precipitates, or DNA extracts from them resulted in the appearance of Vi organisms in initially W types of *Salmonella typhosa*, *Salmonella paratyphi* C, and *Paracolonobacterium ballerup* (125). The mechanism remains unknown. Laigret *et al.* (229) observed that *Proteus vulgaris*, cultivated for 24 hr. in typhus-infected eggs, acquired the antigens characteristic of *Proteus* OX-19 and is agglutinated by OX-19 anti-sera. The acquired antigenic change appears to be stable.

*Morphological variants.*—Tulasne (422) has reviewed much of the literature concerned with the little understood L-forms of bacteria. A method utilizing penicillin in conjunction with high salt concentrations is said to facilitate isolation of L-forms (382). Lavillaureix (237) showed that an L-form retained the high pathogenicity of the vibrio from which it was derived.

In studies of a filamentous mutant of *Candida albicans*, Nickerson & Chung (295, 296) have shown that the genetic lesion is in the cell division mechanism and is probably an impairment of a dissociable metal chelate mechanism which normally couples a flavoprotein oxidation to a reaction essential for cell division.

Lewin (247) has studied a series of 12 *Chlamydomonas moewusii* mutants with paralyzed flagella. No allelism was detected. Certain paralyzed mutants of the + mating type recovered motility after pairing with motile, - gametes and incubation in the dark to suppress cytogamy. Recovery is probably due to passage of cytoplasmic motility factors across the protoplasmic bridge connecting the paired gametes. Certain paralyzed - cells can act as donors of such factors when paired with unlike + paralyzed cells. The paired cells are essentially heterocaryons and can be utilized to provide information on the allelic relations of different mutations causing paralysis. No serological differences were found between motile and paralyzed flagella (274).

A variant strain of *E. coli* B forms flagella-like filaments which cause a viscous drag (62). The efficiency of plating varies for certain of the T phages and the filaments may be phage-specific as indicated by their inactivating effect for certain phages. The filamentous variant resembles the mutants which are semiresistant to phage in a number of respects.

#### RESISTANCE MUTATIONS

*Miscellaneous chemical and physical agents.*—The locus causing inhibition by norleucine has been found to be closely linked to the  $Gal_5^-$  and  $Arab^-$  loci in *E. coli* K-12 (78). Szybalski & Nelson (411) found that *E. coli* B/r resistant to radiation was also resistant to nitrofurans. Four closely linked

alleles near the streptomycin resistance locus were found to determine four consecutive levels of furadroxyl resistance in *E. coli* K-12 (411).

The processes of adaptation and deadaptation of *Saccharomyces cerevisiae* to phenol and thymol have been studied by Wild & Hinshelwood who suggest that the development of resistance is probably not mutational (452). *Saccharomyces ellipsoideus* has been adapted to grow on high NaCl concentrations (464), and strains adapted to high copper concentrations have been shown to accumulate significantly more copper than the parental sensitive strain (287).

Moewus (279) reports that sterility in a *Chlamydomonas* strain is due to synthesis of rutin which acts as a sterility hormone. An enzyme system present in a rutin-resistant mutant or formed adaptively in some formerly sensitive strains converts rutin to an inactive form which antagonizes the rutin inhibition (280).

Concentrations of cobalt approaching 10 per cent were found in cells of a cobalt tolerant strain of *S. cerevisiae* when grown on media containing 750 p.p.m. of the metal (317).

Differences in the temperature tolerance of some strains of *Tetrahymena pyriformis* have been reported by Slater (388).

*Antibiotic resistance.*—A number of papers concerned with the question of the origin of antibiotic resistance appeared during the year. McMurtrie & Berman (269) showed by indirect selection the existence of mutant cells able to grow on 200  $\mu\text{g}/\text{ml}$  of streptomycin in a *Brucella* culture normally inhibited by 1  $\mu\text{g}/\text{ml}$ . About one-quarter of the mutants were streptomycin-dependent. Fully resistant clones of *E. coli* and *Micrococcus pyogenes* var. *aureus* were observed to occur at random on streptomycin gradient plates by Rosenblum (354), whose studies of a number of factors influencing the frequency of occurrence of resistant cells yielded results consistent with a mutation-selection mechanism.

Goldstein (158) presents a number of types of evidence, including replica plating and plate redistribution experiments, which strongly support pre-adaptive mutation as the source of streptomycin-resistant and streptomycin-dependent precursor cells in *E. coli*. Although most of the resistant colonies appeared within 24 hr. incubation on either 10 or 100  $\mu\text{g}/\text{ml}$  streptomycin agar plates in equal numbers, the primary dependent colonies appeared only after incubation for at least 24 hr. on 10  $\mu\text{g}/\text{ml}$  streptomycin agar. The morphology of the primary dependent colonies was abnormal and none developed on 100  $\mu\text{g}/\text{ml}$  streptomycin agar even though the secondary dependent colonies (from cells of the primary colonies) grew on both concentrations with normal morphology. Goldstein suggests that the primary streptomycin-dependent mutants are incapable of colony formation on streptomycin agar until further modification occurs. No evidence is yet available as to whether this modification involves secondary mutation(s) or an adaptive response.

Some extremely interesting results are presented by Szybalski (408) who

observed apparent directed mutation to streptomycin resistance. Suspensions of *E. coli* K-12, *E. coli* B/r, and *Salmonella typhimurium* 6 to 18 hr. old were centrifuged and washed with phosphate buffer 3 to 5 times, then resuspended in buffer in concentrations of  $10^8$  to  $10^7$ /ml. The resuspended cells were incubated overnight at 36°C. to exhaust all possible nutrients, were assayed, and then divided into two parts. Streptomycin in concentrations of 50 to 100  $\mu$ g/ml was added to one part, the other serving as control. The suspensions were incubated for two weeks at 36°C., then assayed on nutrient agar with and without streptomycin (100  $\mu$ g/ml). In successful experiments, 1 to 70 per cent of the cells survived and, in such cases, the majority of the cells surviving in the presence of streptomycin was found to be completely and permanently resistant to the antibiotic. A selective mechanism seems improbable in view of the lack of nutrients, the small size of the populations involved, the rarity of completely streptomycin-resistant mutations, and the fact that most of the streptomycin cells became highly resistant. The absence of selection was confirmed in experiments employing mixtures containing genetically marked streptomycin-resistant *E. coli* cells. Genetic recombination analysis showed consistent segregation and linkage relationships. Szybalski cites similar unpublished results obtained by Zinder with *Salmonella typhimurium* and credits Akiba with the original observations suggesting these experiments.

The finding that most *in vivo* penicillin-resistant staphylococci produce penicillinase whereas *in vitro* isolates do so only rarely could be due to the particular selective environments since 7 out of 313 resistant clones, isolated at random by means of a layering technique, were found to produce penicillinase (358). Kraft & Braun (225) discuss some of the factors influencing the efficiency of isolating antibiotic resistant mutants.

Adaptation of *Aerobacter aerogenes* to acetate and to the drugs propamidine, proflavine, and chloramphenicol have been added to the growing list of microbial adaptations studied by Hinshelwood and his collaborators (94).

The relative penicillin sensitivities of a number of *E. coli* auxotrophs were determined (45) and studies of a glycine-requiring mutant failed to give support to the hypothesis that penicillin interferes specifically with the utilization of glycine for peptide synthesis. The cross resistance of *Bacillus megaterium* and *Mycobacterium ranae* mutants to a number of antibacterial agents permits an arrangement of the antibiotics into cross resistance groups which provide an experimental basis for the design of multiple chemotherapeutic procedures (409, 410).

Bacteria resistant to streptomycin were found to accumulate significantly greater amounts of nucleic acids (39). Resistance to chloramphenicol in *Micrococcus pyogenes* var. *aureus* is accompanied by significant alterations in carbohydrate metabolism (308) and decreased requirements for niacin, thiamine, and arginine (341).

Development of resistance to chloromycetin by salmonellae seemed to be due to mutation and selection (315) and was accompanied by antigenic and morphologic changes.

Almost all isoniazid-resistant mutants of the tubercle bacillus are found to be catalase negative, to have somewhat different growth requirements, and to have lost their virulence for guinea pigs (86, 87, 270, 283). There is some evidence that a corresponding decrease in human pathogenicity does not always occur (398).

Srb (394) has shown that canavanine has an antibiotic effect on yeast which can be counteracted by either D- or L-arginine, or, to a lesser extent, by lysine. Both mendelian and cytoplasmic inheritance of streptomycin resistance in *Chlamydomonas reinhardi* has been reported by Sager (366).

### CYTOPLASMIC FACTORS AND EFFECTS

Genetic investigations of paramecia and yeasts have been especially fruitful in illuminating the role of the cytoplasm in heredity and differentiation. Cytoplasmic effects have recently been studied in a number of other microbial forms which promise to be of value in such investigations.

Reference has already been made to the cytoplasmic motility factors restoring motility to paralyzed gametes of *Chlamydomonas moewusii* (247) and to the cytoplasmic determination of streptomycin resistance in a mutant strain of *C. reinhardi* (366). Sex in *Blastocladiella emersonii*, an aquatic Phycomycete, seems not to be under genotypic control but to be determined by a unilateral transfer of cytoplasmic constituents between copulating cells (73). The postulated "gamma" cytoplasmic factor may be identifiable with particles stainable with the Nadi reagent.

Color phase variation in *Serratia marcescens* (66) seems to be controlled by cytoplasmic interactions with the environment. The direct transmission of the colicinogenic property between *E. coli* strains and between *Shigella sonnei* and *E. coli* suggests an infectious cytoplasmic genetic factor (138).

Serial propagation of *Aspergillus nidulans* by asexual conidia leads ultimately to an imperfect form (215). A heterocaryon between such a somatically selected imperfect form and a yellow-spore mutant sexual strain resulted in all possible types of nuclear fusion in ascus formation. Nuclei of both types behaved in a sexually normal fashion when extracted from the heterocaryon, indicating that the loss of the sexual stage involves an extranuclear change.

Studies concerned with genic and cytoplasmic determination of respiratory mutants in yeasts and *Neurospora* have been referred to in an earlier section (234, 343, 344, 418).

The mate-killing of *Paramecium aurelia* appears to be due to damage to the micronucleus (386). Whereas the kappa-killers in Variety 4 respire at a significantly higher rate than sensitives and seem to utilize a pathway different from the normal cytochrome system, no differences in respiration rates or pathways were observed between isogenic mate-killers and sensitives in Variety 8 (243).

Nanney (289) observed a significantly lower x-ray inactivation dose (37 per cent survival) for kappa than for the corresponding paramecin, indicating that they have a different physical basis. The observation of nearly equal inactivation doses for wild-type and a mutant kappa is incompatible

with the hypothesis that mutation of kappa involves a splitting into two smaller, mutant kappas. A kind of mutual exclusion has been observed between normal kappa particles and inactive "pi" particles which differ from kappa particles both morphologically and serologically (170). The pi particles are believed to have originated by mutation of kappa.

A series of nucleo-cytoplasmic interactions in the determination of mating type in group B varieties of *P. aurelia* has been postulated by Nanney (288).

Sonneborn (390) reviews the literature concerned with the relation of autogamy and conjugation to senescence and rejuvenation in *P. aurelia* and presents extensive data. Conjugation is not essential; periodic autogamy alone can maintain vigor. In the absence of autogamy, the rate of fission declines and death of the line is the ultimate result. Autogamy varies in its effects, depending on the interval since the previous autogamy. Rejuvenation is observed if the fission rate has not declined too far; later, rejuvenation may not occur, the line continuing at the rate before autogamy. If the interval has been too long, autogamy is followed by quick death.

*Phenotypic variation.*—A number of instances of phenotypic changes accompanying the development of drug resistance have already been indicated. Thus, differences in nucleic acid metabolism (39), carbohydrate metabolism (308), requirements for certain growth factors (341), antigenic and morphological characteristics (315), and catalase activity and pathogenicity (86, 87, 270, 283), have been observed in bacterial strains resistant to various antibiotics.

Exposure of *E. coli* to copper ions (437) results in persistent changes to a small colony type, variations in lactose, glucose, and nucleic acid metabolism, and an increase in the resistance to ultraviolet. The phenotypic changes appear to result from an effect of the copper ions on the cytoplasm since cultures inoculated with only 150 cells, most of which would be killed, all produced small colony variants.

A number of adaptations of yeasts and bacteria to phenol and thymol (452), to galactose (114), to acetate and to the drugs propamidine, proflavine, and chloramphenicol (94), are believed to be largely the result of cytoplasmic alterations. Similarly, adaptation of *E. coli mutabile* to lactose was concluded to be nonmutational (95, 96), a conclusion in conflict with the opinions of many investigators of this problem. Stable antigenic variations have been induced in *Salmonella* somatic antigens (65) and in *Proteus vulgaris* (229). Repeated transfer of an avirulent strain of *Mycobacterium tuberculosis* in media containing nonionic surface active agents results in morphological changes which persisted for a number of transfers in normal medium (172).

The virulence of *Candida albicans* for mice is greatly enhanced by simultaneous injection of chlortetracycline, whereas growth of the yeast in media containing chlortetracycline had no effect (355). The increase in virulence appeared to be stable on subsequent repeated transfer on culture media. Some modifications of a synthetic medium for *Podospora anserina* devised



by Tavlitzki (416) provoke morphological modifications in the organism which does not require amino acids or vitamins.

Several additional examples of host-induced phenotypic modifications of bacteriophages have been reported during the year. The plating efficiency of a staphylococcal phage on two host strains was observed to be determined by the host in which the phage was grown (340). Heating the lysate caused the loss of the ability to attack one of the hosts and it is postulated that host control is effected by control of the production of heat labile phage substance required for plaque formation on the second host strain. A lysogenic colony of *Xanthomonas pruni*, isolated from a heavily phaged plate, was found to carry a phage differing from the original virus in host range, plaque morphology, and serology (116). The efficacy of a phage strain in causing inhibition of growth of different host strains was found to be a function of the race of phage and the host strain on which it was grown (429). Hughes & Gots (205), in addition to finding different host ranges after lytic passage of a temperate *E. coli* phage through two different hosts, also observed differences in host range and plaque morphology between phages produced by lytic and lysogenic passage through the same host.

Anderson (14) has continued the analysis of the interesting phenotypic and genotypic host-induced modifications of Vi-phage II which result in the 33 Vi-phage types. In general, phenotypic modifications of the phages result from growth in nonlysogenic Vi-type strains whereas propagation in Vi strains, lysogenic for type determining non-Vi, temperate phages, produces genotypically changed Vi phages which, however, may still be altered phenotypically by growth in nonlysogenic hosts. Structural formulae have been assigned to the 11 known lysogenic Vi types, the validity of which has been demonstrated by the synthesis of Vi phage types indistinguishable from the standard Vi phages by growth of appropriate phages in appropriate nonlysogenic and lysogenic Vi strains.

#### GENE ACTION

*Enzymatic constitution and gene action.*—Additional examples of the close relation between genes and enzymes, as shown by the apparent loss of the pertinent enzyme as a result of biochemical mutations, have been recorded during the year (4, 80, 127, 286, 448). In addition to showing the absence of enzymatic activity in the mutant, Fincham (126, 127) presents accessory evidence strongly indicating that a locus in *Neurospora* is concerned rather directly with the synthesis of a single enzyme. Penicillinase producing penicillin-resistant mutants of staphylococci have been isolated *in vitro* (358), furnishing another example of the apparent synthesis of new enzymes in a mutant strain. Markert & Owen (265) have shown in immunogenetic studies of tyrosinase specificity in *Glomerella* that the amount of enzyme activity is a function of the genotype and environment, that differences in activity reflect differences in the number of tyrosinase molecules synthesized, and that the antigenically and enzymatically active surfaces of the tyro-

sinase molecules are at least in part identical. Serological tests showed that tyrosinases were species specific and no mutagenic effect of antibodies was observed.

A number of characteristics have been observed to be ploidy dependent in yeasts (302, 303), and  $\alpha$ -methyl-glucosidase activity is reported to be ploidy dependent and gene-dosage independent (305). Lindegren & Haddad (250) observed a linear increase in yeast cell volume during budding and suggest that the genome acts at a linear rate in the synthesis of the enzymes required for growth.

Hirsch (188) discusses the ecological implications of his observations that cellulase production in *Neurospora* is temperature dependent.

*Induced synthesis of enzymes.*—One of the most direct approaches to the study of gene action is the investigation of the induced synthesis of enzymes. The rapid advances in this field in recent years have been succinctly reviewed by Campbell (71a). Monod (281) has briefly discussed the role of genetic and other factors influencing enzyme biosynthesis.

The environmental conditions influencing  $\beta$ -galactosidase in *Neurospora* have been studied and the implications for the mechanisms of induction discussed (231). A large difference between the dissociation constant of  $\alpha$ -methyl-glucoside as an inducer and as a substrate leads Spiegelman & Halvorson (391) to suggest abandonment of the complexing concept as an explanation of the role and specificity of inducers. Study of induced  $\beta$ -galactosidase synthesis in uniformly  $C^{14}$  labelled *E. coli* cells showed that less than 1 per cent of the carbon of the enzyme could have been derived from any precursor existing at the moment of induction and convertible to enzyme (357).

The relationships between induced enzyme synthesis and other cellular activities have been studied by a number of investigators. A correspondence was observed between the ultraviolet inhibition of induced maltase synthesis in yeast and protein synthesis as well as nucleic acid synthesis (169).

Sher & Mallette (383) achieved an uncoupling of the induced synthesis of L-lysine decarboxylase of *E. coli* from cell division by inhibiting the latter with sulfur mustard. A similar approach was used by Pardee (313) who showed that purines and pyrimidines were required for induced synthesis of enzymes in *E. coli* auxotrophs requiring these compounds. Inhibition of DNA synthesis by mustard did not prevent induction and synthesis of enzymes. Pardee suggests that continuous RNA synthesis may be essential for protein synthesis and that the RNA present in the bacterial cell may be largely an inert metabolic by-product.

Cohen & Barner (82, 83, 84) have studied a thymine-requiring mutant of *E. coli*, which synthesizes RNA and undergoes cytoplasmic growth in the absence of thymine, under conditions when very little or no DNA is synthesized. Such unbalanced growth establishes a condition unfavorable to cell division even if thymine is later supplied. Induced synthesis of xylose isomerase was observed in such cells in the absence of DNA synthesis, in-



dicating that the cytoplasm is the seat of enzyme induction and synthesis. An interesting observation bearing on the genetic control of induced enzymes is reported by Hotchkiss & Marmur (199) who found that DNA extracts prepared from fully induced and from non-induced mannitol utilizing strains of pneumococcus were equally effective in genetic transformation of mannitol negative mutants to mannitol positive.

Fox (131) reports evidence for a dual genetic control of tyrosinase which appears to be an induced enzyme in *Neurospora*. One genetic system seems to govern the specificity of the enzyme and a second seems to control the concentration. The implications of these findings for protein synthesis are discussed.

An induced enzyme in *Aerobacter aerogenes* appears to be associated with particles having a sedimentation constant of 20 Svedberg units (53).

*Pseudoallelism*.—Reference has been made to the observation of recombination between different mutations at the  $td^-$  locus of *Neurospora* (440). Recombination has also been noted between all tested pairs of adenineless mutants in *Aspergillus nidulans* (336). In this case, closely linked markers on each side of the  $ad^-$  locus prove that crossing-over occurs. Results of transduction experiments in *Salmonella typhimurium* also suggest recombination within groups of presumed alleles (100). All five groups of mutations tested in Pontecorvo's laboratory (328) exhibit crossing-over between mutations which behave as alleles and he considers as confirmed the conclusion that allelism and pseudoallelism are one and the same thing.

### TRANSMISSION GENETICS

Glass (157) has prepared a résumé of a symposium devoted to genetic recombination which included excellent summaries of much of the data on recombination in microorganisms. This symposium will be published as a supplement to the *Journal of Cellular and Comparative Physiology*. A useful reference volume summarizes the knowledge of sexual reproduction in microorganisms (443).

### BACTERIOPHAGES

The analysis of reproduction of the bacterial viruses on the genetic and chemical levels proceeds at a continued rapid pace. In addition to serving as a model virus-host system amenable to precise experimental control, the bacterial viruses are taking on increased significance as evidence accumulates concerning the integration of the genetic systems of the bacteriophages and their bacterial hosts.

*Taxonomy and morphology*.—On the basis of four taxonomic criteria, Adams & Wade (5) consider the coli-dysentery phages T3, T7, and D44 along with two phages for *Serratia marcescens* to constitute a species.

A number of phages have been purified and examined chemically or in the electron microscope. These include the staphylococcal phage K purified by chemical precipitation and filtration (197), and the P1, P2, and P3

phages carried in the lysogenic Lisbonne strain of *E. coli* (228). The temperate phage  $\lambda$  carried in the *E. coli* K-12 strain has been purified and characterized chemically and in the electron microscope (260). A temperate phage from *Micrococcus pyogenes* var. *aureus* was found to be similar to virulent phages (76) but a significantly smaller radiosensitive volume was found for temperate phage  $\lambda$  than for a virulent  $\lambda$  mutant (121).

#### VIRULENT PHAGES

*Invasion.*—A number of papers have been concerned with the initial steps in the phage reproduction process. Additional evidence is presented that T2 is attached to the cell by bonds formed between positively charged amino groups on the virus and complementarily spaced carboxyls on the cell surface (338). A similar mechanism is postulated for T1. Once attached, a leakage into the medium of phosphorus and sulfur from the host cells is observed, due probably to an enzymatic reaction triggered by the irreversible phage attachment (337). Adsorption of T2 or T6 to *E. coli* B cell membranes results in conversion of membrane nitrogen to a nonsedimentable form in amounts proportional to the ratio of virus particles to membranes (30). The influence of the ionic composition of the plating medium on the viable cell count of infected cells in which the virus is not yet irreversibly attached has been studied (263). The initial steps in infection with T7 are similar to those observed with T1 (261); the initial adsorption is sensitive to the ionic environment and it is followed by an irreversible binding which is dependent on the temperature but independent of the host cell metabolism.

Weidel and his associates have continued their studies of *E. coli* B cell membranes and virus receptors (438, 439). Dissociation in 90 per cent phenol uncovers a lipopolysaccharide with receptor activity for T3, T4, and T7. This layer contains a heptose which is absent in similar preparations prepared from mutants resistant to T3, T4 and T7 which show no receptor activity (439). Weak alkali extracts a lipo-glycoprotein which shows receptor activity for T5. A picture of the entire cell membrane is presented, consisting of an innermost lipopolysaccharide layer with receptor activity for T3, T4, and T7, covered by a lipo-protein layer with T2 and T6 receptor activity with the discontinuously distributed, loosely bonded T5 receptor substance on the surface (438).

Calcium or magnesium ions in adequate concentrations are required for penetration of T5 after adsorption and for a full phage yield (255). T5 adsorbed without penetration is not neutralized by antisera, causes disintegration of the bacterial chromatin, and interferes with growth of other phages.

The specific reversible inhibition of T2 by adsorption of an inhibitor of bacterial origin can be relieved by a variety of treatments, including heating, which may account for the anomalous results of thermal inactivation kinetics sometimes observed (368). Anomalous results in studies of anti-

serum neutralization of T2 (72) also appear to be due to T2 inhibitors of bacterial origin.

Ribonuclease and chymotrypsin prevent the permanent attachment and multiplication of adsorbed *Rhizobium* bacteriophage (223, 224). Chymotrypsin treatment of cells or phage separately has no effect and the enzyme is effective in preventing permanent attachment only during the first minute after adsorption.

*Intracellular growth of phage.*—Sections of *E. coli* cells infected with T4 have been examined in the electron microscope in a study of the nuclear changes resulting from phage infection and growth (258). French (140) employed T2 labeled with  $C^{14}$  to show that only about 1 to 2 per cent of the parental phage protein label appeared in the progeny, thus confirming the earlier observations of Hershey & Chase (179) using  $S^{35}$  labeled phage. Studies of the distribution of  $P^{32}$  following infection with labeled T2 showed that, during the first half of the latent period, the largest fraction of the  $P^{32}$  apparently remained in fibrous DNA structures with most of the remainder broken down to low molecular weight substances (433). During the second half of the latent period, some of the  $P^{32}$  was reincorporated into mature particles. None of the  $P^{32}$  was found in structures intermediate in size between DNA fibers and mature phage particles (433). Studies of T7 labeled with  $P^{32}$  and  $N^{15}$  (261) showed T7 to be roughly similar to T2 and T6 in the transfer of the labeling to progeny nucleic acid and protein. Irreversible attachment of T7 is followed by a release of some virus P to the medium and then by destruction of the integrity of the virus particles. It was concluded that the transfer of T7 parental material to the phage progeny is largely nonspecific and is largely uncorrelated with the transfer of hereditary properties (261). Isotope competition experiments with T2, however (180), indicate that transfer of parental DNA to progeny phage probably occurs in larger, possibly genetically active pieces.

Volkin (426) has shown that the DNA fraction of osmotically ruptured T4 phage is associated with a protein which comprises about 25 per cent of the total phage protein and which presumably would be injected into the host cell along with the phage DNA. Considered along with a possibly low amount of specific DNA transfer and the 1 or 2 per cent of protein transfer, Volkin's finding indicates that the transfer of phage genes could involve protein as well as DNA transfer.

Abortive infection of a T2 strain in starved washed *E. coli* K-12 cells has permitted a study of the nutritional requirements for T2 reproduction in this bacterial strain (166, 167).

Chloramphenicol, known to inhibit bacterial protein synthesis with little effect on DNA and RNA synthesis, was found to inhibit phage maturation when added at any time during the growth cycle. If it was added in the latter half of the latent period, lysis of the cells occurred and mature phage particles approximating the number present at the time of addition of the drug could be demonstrated (55). Increased phosphate concentration in a

glucose-salts medium relieves the inhibition of glucose and phosphate utilization and DNA synthesis by proflavine and permits T2 phage yields of up to 10 per cent of normal (262). Undiluted lysates of a *Staphylococcus aureus* phage were found to inhibit lysis by a number of other phages (195). The inhibitor was shown to be associated with particles of the same size as phage and to be destroyed by heating at 52°C. for two min., a treatment without effect on the lytic particle titer.

Attempts to demonstrate T3 or T4 neutralizing antigen during the vegetative stage of intracellular growth failed (31). Proflavine-inhibited T4-infected cells synthesized normal amounts of neutralizing antigen, the proflavine seeming to inhibit the attachment of the antigen to the immature virus. Complement-fixing antigen appeared in T5-infected cells a few minutes before infectious phage particles and, during the remainder of the latent period, was synthesized at a higher rate than infectious phage (232). Barner & Cohen (27) have presented further data on the synthesis of thymine induced by T2 infection of a thymine-requiring mutant. Cells infected in the absence of thymine synthesize DNA as rapidly as if it were present and a net synthesis of thymine, 5-hydroxy-methyl cytosine, adenine, and guanine was observed. Comparison of the rate of synthesis of the bases with their molar ratios in phage DNA indicated that only phage DNA was accumulating in the infected cells (27).

*New phage systems.*—A vibrio-phage system has been described which produces lysogenic cells differing from the normal in growth habit (389). Four phages active against *Rhizobium meliloti* (314) have been isolated and characterized. Initial steps of infection with another rhizobium phage appear to be similar to those of the coliphages T1 and T2 (223, 224). Tests of genetic recombination in the small phages, S-13 and Phi-X-174, have as yet been negative (466). Some success has been attained in purification of these small phages.

*Recombination in phages.*—Levinthal (245) has elaborated his hypothesis that the heterozygotes observed in recombination experiments with T2 are intermediates in the formation of recombinants. The chief experimental evidence for this view is the finding that, in 3-factor crosses, particles heterozygous for the middle marker are generally recombinants for the end markers. Although their mode of formation is unknown, Levinthal presents calculations showing that frequency of heterozygotes is sufficiently high to account for the formation of all of the recombinants. This enlargement of the Visconti-Delbrück hypothesis (423) of phage recombination seems to harmonize the most seriously conflicting data.

The investigations of recombination in T1 have been continued by Bresch & Menningmann (61) who now have shown that all seven of the mutations so far studied fall into the same linkage group. Recombination phenomena in T1 seem consistent with the Visconti-Delbrück hypothesis although the number of rounds of mating cannot be estimated from the present data. Heterozygotes have apparently not been observed. Negative results were obtained in recombination tests with S-13 and Phi-X-174 (466).

Some extremely intriguing preliminary results reported by Hershey *et al.* (180) suggest that the apparently simultaneous mutation of several loci observed at quite high frequencies in T3 may actually be due to genetic recombination between the phage and the host chromosomes. Premature lysis of phage-host complexes which produce the T3 mutants showed equal proportions of two mutant phenotypes, suggesting that the mutants must be formed in pairs by a single event. Independently maintained cultures of *E. coli* B could differ genetically and hence on the recombination hypothesis could give different results in "crosses" with T3. Whereas the total frequency of mutants produced did not vary significantly among six such bacterial strains, the proportions of the various phenotypes did vary significantly. Serial back-crosses to the same parent ultimately result in near identity of parental and back-cross progeny genotypes. Hence repeated "back-crosses" of T3 to the bacterial host should produce identical homologous portions of the genetic material in host and phage and, on the recombination hypothesis, mutant phages should no longer be produced. This was realized in experiment and, to further support the hypothesis, mutant phages were again produced when these "back-crossed" phages were "out-crossed" to a nonhomologous host.

#### LYSOGENY

Attention is again drawn to the extensive and beautiful review by Lwoff (256) who discusses the historical development, present state of knowledge, and the biological significance of lysogeny.

*The lysogenic cycle.*—Infection of a bacterium with a temperate phage may be productive with subsequent phage production and lysis, reductive with the establishment of a lysogenic clone, or, more rarely, abortive, which may result in either death or survival of the infected cell. A number of papers have been concerned with the "decision" governing the outcome of the infection. Lwoff *et al.* (257) have extensively studied lysogenization of *Salmonella typhimurium*. The probability of a reductive response increases with the multiplicity of infection. It is decreased by ultraviolet irradiation following infection, by anaerobiosis, by cyanide treatment, by heating at 42°C., and by various substrates such as lactic, pyruvic, and succinic acids. The agents influencing the response to infection do not exert their maximum effect until 6 min. after the infection and have no effect after 9 min. Inhibitors, such as 2,4-dinitrophenol, added at the time of infection, increase survivors to 100 per cent after about 30 min. The survivors are not lysogenic and are sensitive to the phage (257). Use of synchronously dividing salmonella revealed differences in the frequency of lysogenization of cells infected at various times during the division cycle (233, 259).

Bertani & Nice (42) have shown that exposure to 20°C. blocks the lytic development which would occur at 37°C. in *Shigella dysenteriae*, strain *Sh*, infected with the temperate phage P1 or its virulent mutant. The temperature-sensitive period occurs after the first 20 min. but before any mature intracellular phage is formed. *Sh* cells infected with P1 become lysogenic

at 20° more frequently than at 37°. No temperature effect was observed for the temperate phage P2, or for the virulent phages of the T1-T7 group (42).

The response of sensitive *E. coli* K-12 cells following infection with  $\lambda$  and with one of its virulent mutants has been studied (350, 351). Lysogenization of *Streptomyces griseus* has been studied and a high frequency of non-lysogenics observed in lysogenic cultures (441).

In an important paper concerned with superinfection of lysogenic shigella with temperate mutants of the carried phage, Bertani (41) discusses the concept of preprophage. This term signifies a nondividing stage of the superinfecting phage which is passed on to either daughter cell at division, and which may become a prophage in cases of double lysogenization or prophage substitution, or may become a vegetative phage in those cells shifting to the lytic cycle. In an appendix, Bertani develops equations permitting a quantitative test of the preprophage hypothesis in single burst experiments.

The spontaneous liberation of phage has been observed in a lysogenic *Pseudomonas aeruginosa* (101) strain in which the lysogenic phage and a virulent mutant were shown to have similar latent periods and initial steps in the infection. Beumer (43) has made extensive studies of the influence of various media and environmental factors on the spontaneous liberation of three distinct phages by the Lisbonne strain of *E. coli*.

The induction of  $\lambda$  phage synthesis and lysis by ionizing radiations is the basis for a quantitative biological test for low doses of radiation (264). Franklin (132) observed a maximum at 2650Å for monochromatic ultraviolet induction of lysogenic *E. coli* K-12. Jacob & Wollman (211) have shown a spontaneous induction of  $\lambda$  development in crosses between Hfr lysogenic and F- nonlysogenic strains. In a subsequent paper (458) they show that this spontaneous induction modifies the segregation of characters linked to lysogeny. The initial nuclear changes are similar in lytic and lysogenic infections of *Shigella dysenteriae* and consist of a condensation of the chromatin (447). In lysogenization, the condensed chromatin is reorganized into nuclei indistinguishable from those of nonlysogenic cells.

*Genetic recombination in lysogenic phages.*—Wollman & Jacob (210, 457) have published extensive studies of recombination in the vegetative stage among  $\lambda$  mutants which indicate that the mechanism of recombination is very similar to that of the virulent T2 coliphage. Mutants differing in virulence, plaque size and type, and ability to lysogenize were employed. Recombination can be realized by simultaneous infection of sensitive cells or, more readily, by superinfection of lysogenic cells previously induced by ultraviolet. Heterozygotes were observed. All 16 of the mutations studied are in only one linkage group and a tentative linkage map with nine reasonably accurately located genes is presented. Studies of recombinant frequencies in 3-factor crosses at the time of lysis and during the latent period

reveal still further similarities to the mechanism of recombination in T2. The chief difference appears to be the smaller number of cycles of mating in  $\lambda$  recombination.

A second mechanism of genetic recombination in  $\lambda$  phages has been discovered by Appleyard (17) in studies of a doubly lysogenic *E. coli* K-12 strain produced by superinfection of a defective lysogenic strain carrying a prophage which was almost entirely unable to produce infective phage. The two prophages of the double lysogenic carry six recognizably different prophage alleles. Most doubly lysogenic cells produce, after induction, mixed yields of genetically different phages. The double lysogenic strain produces bacterial segregants which are singly lysogenic, and which were shown to have lost one or three prophage alleles due, apparently, to a recombination of prophage genes during bacterial growth by a process similar to somatic crossing over.

*Genetic segregation of lysogenicity.*—Additional evidence strongly indicating a chromosomal site for the prophage has been published during the year. Jacob & Wollman (211, 458) show that segregation of *gal*<sub>4</sub> and *lac*<sub>1</sub> is markedly influenced by spontaneous induction in the zygotes resulting from the Hfr or F+ lysogenic crossed with F- nonlysogenic strains. Similar differences between reciprocal crosses of F+ with F- lysogenics and nonlysogenics have been observed by Frédéricq (137) who also confirmed the linkage between *gal*<sub>4</sub> and lysogeny. The lysogeny-*gal*<sub>4</sub> linkage was also observed by E. Lederberg (238) in crosses with four independently isolated, fertile  $\lambda$ -sensitive strains which also revealed a second locus, Np, which modifies the expression of the lysogenic locus, Lp. The  $\lambda$  prophage appears to remain fixed to the Lp locus and, although differences in frequencies were observed in crosses reciprocal with respect to the F compatibility factors, the same types of recombinants were observed.

Bertani's estimate (41) of 2.2 prophages per lysogenic *Shigella* cell, which is close to the average number of nuclei per cell, is also compatible with a hypothesis of a chromosomal site for the prophage.

Extensive data concerned with the segregation of lysogenicity during genetic recombination in *E. coli* K-12 is discussed by Appleyard (16). Crosses between single lysogenics carrying distinguishable prophages did not produce double lysogenics or nonlysogenics. The linkage to galactose was again confirmed. Similarly, in crosses of a double lysogenic *gal*+ F- with a nonlysogenic *gal*- F+ strain, nonlysogenicity and *gal*- were closely associated, showing that double lysogenicity is also linked with *gal*. Appleyard's data indicate a genic component of lysogenicity. This component is complex; in addition to determining lysogenicity, it embodies prophage genes which recombine in the segregation of monolysogenics from double lysogenics (17). Although this genic component could be a complex of bacterial genes, one controlling each prophage characteristic, Appleyard prefers the hypothesis of an additional cellular component, the prophage,



attached at a specific chromosomal site, since this hypothesis can more easily explain the segregation of prophage genes (17) and the production of non-lysogenics by long exposure of lysogenics to ultraviolet.

*Characters associated with lysogenicity.*—In view of the conclusion that the prophages responsible for lysogenicity occupy a chromosomal site and hence may be regarded as a part of the genotype, it is understandable that lysogenization is sometimes accompanied by changes in apparently hereditary traits. The best known example is the conversion of *Corynebacterium diphtheriae* from nontoxigenicity to toxigenicity by lysogenization with certain temperate phages. During the year, additional convincing evidence has been supplied indicating that lysogenization *per se* is responsible (26, 164, 165). Groman (164, 165) has shown the conversion to toxigenicity is phage specific and the  $\beta$  temperate phage responsible for the conversion has been shown to behave in a fashion quite typical of temperate phages (26). These conclusions have been largely confirmed by Hewitt's observations (181, 182, 183) of a different *Corynebacterium*-phage system, which has been studied more particularly from the viewpoint of the influence of phages upon bacterial evolution.

Boyd (54) discusses the parallel between the immunity to other phages conferred by lysogenization with certain temperate *Salmonella typhimurium* phages and genetic mutation to phage resistance. The determination of the Vi lysotype of lysogenic *S. typhi* by the carried phage has been further analyzed in detail by Anderson (14). While considering phage typing, it may be mentioned that the Vi+ type A group has been divided into nine subtypes and group I and IV into eight subgroups by the use of a new series of typing phages (297). Other studies of phage typing show the feasibility of classifying salmonella strains by typing phages which correspond in range to the O antigenic groups (432) and the isolation of a series of phages which may be useful in typing certain pasteurellae (349).

*Bacteriocins.*—Lwoff (256) reviews briefly the salient features of the bacteriocins, which may be proteins similar in action to the tail antigen of T2 coliphage.

Frédéricq (136) has studied ultraviolet induction of colicine synthesis in nonlysogenic strains of *E. coli* and finds that, in contradistinction to lysogenic, colicine-producing strains, colicine production begins immediately after induction and ceases after about 2 hr. with no true lysis of the cells. The induction, synthesis, release into the medium, adsorption, and toxicity of pyocine was studied by Jacob (208) who points out that no direct proof exists of a relationship between the bacteriocins and bacteriophages. A new bacteriocin, megacine, was found to be produced by a *Bacillus megaterium* strain (207).

Frédéricq observed transduction of the colicinogenic property between *E. coli* and *Shigella sonnei* (133) with conservation of all of the genetic characters of the transduced strain, and noted that transduction was independent of lysogeny (134). However, he found that the F compatibility



factor of *E. coli* K-12 was involved (135), since no transduction of the colicinogenic property was observed between F- strains.

Studies of a nonreproducing lysine produced by *E. coli* strains following lysis by a virulent phage have not disclosed its nature or mode of action (309, 310).

#### ANIMAL VIRUSES

Research with the animal viruses is largely outside of the scope of this review. A number of publications of interest to the readers of this review should be mentioned, however, for they reflect the value of bacteriophage investigations as prototypes for studies of less easily studied viruses. The problem of virus nomenclature is discussed by Andrewes (15) and binomial names are suggested for some animal viruses.

Dulbecco & Vogt (108) have extended the plaque formation technique to poliomyelitis virus and have isolated pure lines from single plaques. The plaque counting method was used to establish one step growth curves of Western equine encephalomyelitis (109) and Newcastle's disease viruses (244).

Burnet *et al.* have published extensive studies of genetic recombination in influenza viruses (67, 68, 249, 318). Heat-inactivated virus can participate in recombination (67). The observation of double neutralization led to the hypothesis of a normal diploid constitution with the factors studied distributed in two linkage groups (68). Crossing-over has not been observed. Two reciprocal recombinants can be crossed to yield the original parental types (318). A wide range in neuro-pathogenicity was observed among recombinants from a neurotropic and nonneurotropic cross (249). This suggests multiple genic control and Burnet & Lind (69) present an hypothesis of the genetic control of virulence which is somewhat unusual from the orthodox genetic viewpoint.

These recombination studies with the influenza virus take on added interest in the light of Ada & Perry's finding (3) that the virus contains little or no DNA, and that the RNA content is less than 1 per cent.

Granoff & Hirst (163) have observed "phenotypic mixing" in mixed influenza A and Newcastle disease virus infections. Mixed infections of influenza A and B viruses also give doubly antigenic particles suggesting "phenotypic mixing" and, in addition, apparently heterozygous diploid particles producing two types of progeny were observed (159).

#### BACTERIAL GENETICS

*Heterothallism in bacteria.*—The discovery of the F compatibility factors in *E. coli* K-12 strains led Calef (71) to retest for sexual recombination in *E. coli* B strains on the supposition that earlier failures were due to attempted F- $\times$ F- crosses. His observation of recombination between *E. coli* B, which acts as F-, and *E. coli* K-12 F+ strains, paves the way for genetic analyses of the widely studied *E. coli* B strain.

The F polarity of the parents is known to influence the segregation of the unselected markers in *E. coli* K-12 crosses: the genotype of the F- parent is predominant in the recombinant, and a chromosome segment from the F+ parent carrying lysogeny, *gal*<sub>4</sub> and certain other markers is found only rarely in the recombinants. The suggestion has been made that the spontaneous induction of lysis of the recombinants receiving this segment in crosses of F+ lysogenic  $\times$  F- nonlysogenic may be partly responsible for these anomalous results (137, 211).

Nelson & Lederberg (291) show that the elimination of the chromosome segment occurs post-zygotically. Although 80 per cent of the nondisjunctional diploids from crosses involving the *het* factor retain the Mal-S segment from the F- parent, 20 per cent retain one or both markers from the F+ parent, indicating that the primary zygote is genetically complete and that the F+ segment is preferentially eliminated by a postzygotic process.

The influence of F polarity on segregation necessitates a re-examination of linkage relationships. Clowes & Rowley (79) present extensive linkage data, involving a number of strains with a number of different selected and nonselected markers, which suggest a single linear chromosome.

An interesting observation of the contribution of factors from three parents to the genotype of recombinants in *E. coli* K-12 was made by Frédéricq & Betz-Bareau (139). The proportion of tri-parental recombinants was so high that the possibility of successive matings would seem to be eliminated.

A system of genetic exchange in *Serratia marcescens* similar to that in *E. coli* K-12 has been discovered by Belser & Bunting (40). Transduction by filtrates has not been observed and streptomycin treatment of either parental strain is without effect (40). Tests with *Proteus* species revealed no evidence of recombination (206).

**Transformation.**—Some cells of pneumococcus colonies growing in semi-solid agar containing transforming agent may undergo transformation. Determination of the frequency of such colonies containing transformed cells permits quantitative studies of autogenic and allogenic capsular transformations (345). Below a saturating concentration, this frequency is a function of the transforming DNA concentration. Ravin (345) showed that the same DNA extract can cause apparently independent autogenic and allogenic transformations depending on the physiological state of the recipient cells. Abnormal capsular transformation has been observed when cells of certain R strains derived from type II pneumococci are exposed to transforming agents from fully encapsulated type III cells (37). In addition to fully encapsulated type III and intermediate type III cells, a third specific type which is neither II or III has been obtained.

The finding that drug resistance could be conferred by transforming extracts provided a powerful tool for quantitative studies of pneumococcal transformation and has enabled Hotchkiss (198) to demonstrate a cyclical behavior of transformability which is probably related to the division cycle.

The cycles were accentuated by a synchronization of cell division produced by incubating a growing 37°C. culture for 15 min. at 22 to 25°C. after which incubation at 37°C. was resumed.

The screening methods available for streptomycin resistance and mannitol utilization have also enabled Hotchkiss & Marmur (199) to prove that double transformations of these characters occur with a frequency significantly greater than that expected for coincidence of two independent transformations. Another pair of mutations, streptomycin resistance and sulfanilamide resistance, also exhibit linked transformations. The possibility of a spurious linkage due to an aggregation of independent transforming agents during the DNA extraction was excluded (199). When it is recalled that the transforming DNA extracts are highly purified and contain no demonstrable protein, these results are of obvious significance in relation to the ultimate structure of the genetic material.

Investigations of the *Haemophilus influenzae* transformation system have been continued by Alexander, Leidy, Zamenhof and their associates (7, 472, 473). Eight transformable characteristics, varying widely in their frequency of transformation, are known (7). The frequency of a given transformation is proportional, within limits, to the transforming DNA concentration. In further analogy to the pneumococcal system, the frequency of susceptible cells varies during the growth cycle, and there is an exclusion of DNA's since induction of one type specificity or streptomycin resistance can be completely prevented by pretreatment with another type-specific DNA. A correlation between carcinostatic activity and inactivation of transforming agent was observed with a series of nitrogen mustards; biologically active DNA, surviving the mustard treatment, was rendered labile to heat and storage (473). A similar labilization was produced by heating (472). It was shown to be nongenetic, since cells transformed by labilized DNA yielded stable transforming extracts. The parallel to the postulated unstable state of genes after mutagenic treatment but prior to actual mutation is pointed out. Attempts to incorporate 5-bromouracil into the transforming DNA of *H. influenzae* have been unsuccessful (468).

**Transduction.**—A second form of genetic recombination has been shown to operate in *E. coli* K-12 with Morse's finding (282) that the group of  $gal^-$  loci could be transduced to  $gal^+$  by  $\lambda$  lysates of wild type or nonhomologous  $gal^-$  cells. Apparently only the  $gal^-$  loci are transducible.

In studies of the occasional simultaneous transduction of the Fla genes causing flagella formation and of an antigen determining gene, HI, Stocker (400) has obtained evidence for linkage and a linear order for certain genes in salmonella. Demerec and his associates (100) likewise report evidence of linkage of transducible, auxotrophic mutations in *S. typhimurium*; these workers are utilizing transduction in large scale studies of allelic relationships and linkage among auxotrophic mutations. A study of the delay in phenotypic expression of transduced genes in salmonella has been initiated (456).

## FUNGI

*Ascomycetes*.—An extensive consideration of the estimation of linkage, a summary of cytogenetic knowledge and of all of the published linkage data, and a series of linkage maps for *Neurospora crassa* can be found in the paper of Barratt *et al.* (28). A criterion for independence based on the ratio of nonparental, ditype tetrads to tetratypes in unordered tetrads is discussed. In this connection, Whitehouse (445) considers the analysis of unordered tetrads segregating for a lethal or other epistatic factor, applying his formulae to an example from *Chlamydomonas*. A partial linkage map of 11 mutations in linkage group D of *Neurospora* has been published (275). Newmeyer (293) has shown the utility of a plating method for testing allelism or estimating linkage of biochemical mutations in *Neurospora* based on colony counts from ascospores seeded onto minimal and supplemented media. The ratio of 2, 3, and 4 strand double crossovers among 847 completely analyzed asci was found not to differ from the expected 1:2:1 ratio when corrected for nuclear transposition (204).

The formation of protoperithecia, tyrosinase, and melanin in *Neurospora* can be inhibited by incubation at 35°C., by increasing the amount of nitrogen in the medium, or by the presence of tyrosinase inhibitors (187, 189). It is uncertain whether tyrosinase and melanin formation have a causal relation to protoperithecia formation.

Pittenger (319) has shown that the apparently disomic pseudo-wild type strains occur with quite high frequencies in crosses involving closely linked or possibly allelic genes in each of the five linkage groups studied, including the chromosome carrying the mating-type locus. Among 654 wild type progeny derived from such crosses, 99 proved to be pseudo-wild types.

The relation between the nuclear ratio and the growth rate of *Neurospora* heterocaryons varies, depending on the particular biochemical mutants making up the heterocaryon and on the initial nuclear ratio (320). The results indicate that in many heterocaryons there is no selection toward a nuclear ratio giving a maximal growth rate. Holloway (194) has found that at least four and possibly five genes are concerned in the genetic control of heterocaryon formation in *Neurospora*. In addition to determining whether or not a heterocaryon is formed, the genes influence the rate, time of onset, and maintenance of heterocaryotic growth.

Olive (306) discusses the heterothallic behavior of supposedly homothallic fungi and suggests that the term "amphithallism" be applied to such species. He has analyzed such behavior in *Sordaria fimicola* (307) where a spore-color mutant can be utilized to identify crossed perithecia.

Wheeler (444) has summarized the data on the genetic control of heterothallism in *Glomerella*, where a number of mutations are known which block the sexual process at different stages. The multiple factor control of incompatibility in *Podospora anserina* by six pairs of genes has been analyzed by Esser (124). Three of the six pairs also influence the determination of the barrage phenomenon between strains of this fungus.

*Basidiomycetes*.—The formation of monocytons from dicaryotic strains of *Coprinus lagopus* has been observed (277). The mutant component is generally lost. Comparisons between the growth rate of monocyotic strains and the dicaryons formed from them show the dicaryon to be intermediate (278).

Hemi-compatible heterocytons of *Schizophyllum commune* derived from two homocytons having common factors at the A incompatibility locus have been studied by Raper & San Antonio (342). Two different heterocytons having reciprocally unequal nuclear ratios are formed and mutual complementation of nonallelic biochemical deficiencies is somehow prevented in the heterocyton even though dicaryons formed between the same parental strains show complete complementation.

*Extra-sexual reproduction in fungi*.—The investigations of mitotic or parasexual recombination in the filamentous fungi were discussed at some length in last year's review (392) and have been extensively reviewed by Pontecorvo (326), who, with his associates, has largely developed this very important phase of microbial genetics.

The general features and broad implications of parasexual recombination have again been considered by Pontecorvo (327). The analysis of mitotic recombination in *Aspergillus nidulans* has shown two processes to operate: somatic crossing-over in diploid cells and a random reassortment of whole chromosomes in the formation of haploid nuclei (329). The frequencies of mitotic crossing-over which leads ultimately to homozygosis, of haploidization, and of the inverse process of fusion between unlike nuclei are of the order of  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-7}$  respectively. The ultimate result of growth of a heterozygous diploid is, therefore, formation of a number of haploid recombinant strains without a sexual cycle (327). Close agreement was noted between chromosome maps of *A. nidulans* based on meiotic and mitotic crossing-over (330). Further extension of the data on mitotic recombination in *Penicillium chrysogenum* (331, 380, 381) has fully confirmed the earlier conclusions based on studies with *Aspergillus*. The practical aspects of mitotic recombination were also noted (331).

Additional, somewhat more direct evidence of the transfer of genetic incompatibility factors between the nuclei of a dicaryon of a basidiomycete has been supplied by Papazian (312). Little is known concerning the mechanism.

*Yeasts*.—Winge & Roberts (453, 455) have made an important contribution to the explanation of irregular segregations in yeast tetrads. Briefly, they show by genetic and cytological means that supernumerary mitoses occur in the asci of certain strains and that, since some of the nuclei may not survive as spores, abnormal ratios may develop. In addition, binucleate spores may be formed giving rise to directly diploidized and often heterozygous single spore cultures. It is shown that in tetrads containing three dominant and one recessive spore, one of the dominants is usually a heterozygous diploid. Winge & Roberts reject the gene conversion theory as an

explanation of irregular segregations. Other mechanisms such as a cytoplasmic factor or overlapping of generations which could result in aberrant segregation ratios are discussed.

Pomper *et al.* (325) were able by use of an adaptation of the prototroph selection method to isolate prototrophic diploids, triploids, and tetraploids from crosses between complementary, auxotrophic haploids, between haploids and diploids, and between diploids, respectively. The degree of ploidy was confirmed by studies of the kinetics of x-ray inactivation. Ascospore analysis showed that the triploid segregated to form two diploid and two haploid spores at meiosis. A tetraploid yielded four diploid spores.

Polygametic zygotes were suggested as a possible mechanism whereby polyploid zygotes could be formed (6). A number of characteristics including cell size and growth habit (420) and various physiological traits (302, 303, 305) were shown to be ploidy-dependent.

Leupold & Hottinguer (242) analyzed segregation data from asci heterozygous for mating type and a number of growth factor requirements; linkage relationships were calculated. Two cases of complex segregation were apparently due to tetraploidy, and two interacting factors were postulated for complex segregation of pigment formation in *ad<sup>-</sup>* strains and of galactose fermentation. Douglas & Condie (104) have identified two genes which influence galactose fermentation. James (213) has found three complementary loci influencing galactose fermentation.

Complications arising from the persistence of the dicaryon formed by plasmogamy and from the mixture of original gametes and hybrid cells in the mass mating technique are discussed by Lindegren & Lindegren (251).

The relationships between a number of yeasts of the genus *Candida* have been clarified by the isolation of mating types and crossing experiments (450).

Somatic crossing-over induced by ultraviolet is postulated as the mechanism responsible for sector formation in a diploid yeast heterozygous for three complementary genes governing galactose fermentation (213). On the basis of unusually high frequencies of recombination in crosses involving certain linked genes, Lindegren & Shult (252) suggest that crossing-over occurs in the two strand as well as in the four strand stage.

*Algae.*—In *Chlamydomonas*, Lewin (247) has observed a cytoplasmic interchange of motility factors through the protoplasmic bridge joining the paired gametes. Such pairs of copulating gametes, which can be regarded as heterocaryons, were used to provide information on the possible identity or nonidentity of mutant genes (247). Both mendelian and cytoplasmic inheritance for streptomycin resistance was observed in *C. reinhardi* (366).

Exposure to light in the absence of a nitrogen source is necessary for differentiation of cells into gametes in *C. reinhardi* (367). No light is required for the gametic differentiation of nitrogen-depleted cells and the carbon to nitrogen ratio required for gamete formation varies widely depending on the nitrogen level on which the cells were grown (367). Gamone effects have been investigated in *C. eugametos* (129, 130).



Cytological studies of *Chlamydomonas* are reported to reveal a relatively orthodox mitosis and meiosis (375). Chromosome numbers are given as  $36 \pm 2$  for *C. moewusii*,  $18 \pm 2$  for *C. reinhardi* and  $16 \pm 1$  for *C. dysosmos*.

Starr (397) has studied the inheritance both of mating type and of a lethal gene causing lysis of the zygote in a desmid. Only two, nonsister nuclei survive following meiosis in this form. Both mating type and the lethal usually segregate at the first division, and the two factors do not appear to be linked.

The opportunity for physiological genetic studies afforded by the unusual association of the four haploid products of meiosis in *Volvox* has been pointed out by Papazian (311), who has also examined the number of genotypes possible for up to four heterozygous factors.

#### SLIME MOLDS

A summary of the present knowledge of the physiology of the developmental cycle, the nutrition and genetics of the amoeboid slime molds has been prepared by Sussman (405). Synergistic interactions permit certain mixed populations of deficient strains to complete the morphogenetic cycle (406). If a passage of material between the deficient strains is responsible for the synergism, it seems to occur by cell contact (407). No genetic recombinants have been observed among the spores obtained from such mixed populations and, in certain cases, antagonisms have been observed between two deficient strains. Histidine stimulates and adenine decreases the sensitivity of the chemotactic process resulting in the aggregation stage (57). The effect is quite specific, since closely related compounds have no effect on the aggregation stage.

#### PROTOZOA

A new variety of group A of *Parmecium aurelia*, variety 9, containing mating types XVII and XVIII has been isolated (35). The structure and development of the macronucleus have been studied by phase microscopy, and thin sections of mitochondria of *P. aurelia* have been investigated with the electron microscope (115, 333). The mitochondria are similar in structure to the kinetosomes near the bases of the cilia and to the young nucleoli of the macronucleus.

A method of synchronizing cell division of *Tetrahymena piriformis* by temperature treatments is highly effective (376); 85 per cent of the cells divide simultaneously. Cytological studies of *Tetrahymena* during conjugation disclose five chromosomes undergoing a rather normal meiosis with only one of the products of meiosis surviving (346). Corliss (88) has prepared a useful summary and guide to the literature on *Tetrahymena* containing, through 1953, about 500 references in more than one hundred journals.

#### POPULATION GENETICS AND EVOLUTION

A symposium has been devoted to the consideration of speciation and variation in the asexual fungi (273). The papers include an extensive survey

of natural and induced variation in several genera and discussion of species definitions and criteria. The use of bacterial traits subject to simple mutational changes for taxonomic purposes has been discussed (58, 301), and it is suggested that the recognized species of *Brucella* are in reality only varieties or the most frequently occurring biotypes of one species.

Braun and his associates have continued to study the influence of DNA upon population changes in *Brucella* (59, 60). Two effects are noted: a transformation-like effect when certain strains are treated with heterologous DNA; and a non-specific selective effect. The latter is related to a compound produced by mutant cells in the presence of DNA and DNAase.

A differential inhibition of smooth and rough *Brucella* by D-asparagine may exercise a major selective influence through interference with pantothenate synthesis in smooth cells (13). The effect of  $\alpha$ -alanine in favoring the establishment of nonsmooth cells in *Brucella* populations also appears to be related to an inhibition of pantothenate synthesis or utilization (271).

Hewitt has considered the evolutionary aspects of the bacterial virus-host cell interrelationship in lysogenic systems (181, 182, 183). The convincing evidence of an intimate relationship between the genetic material of lysogenic phages and that of the host has been discussed earlier. Preliminary evidence of possible genetic recombination between a supposedly virulent phage and its host (180) indicates that the genetic systems of all bacteriophages and their hosts may be integrated to some extent. Such implications add new interest to the consideration of evolutionary mechanisms in bacteria, and focus attention on the problem of the evolution of host-phage systems.

#### NATURE AND PROPERTIES OF THE GENETIC MATERIAL

In recent years, the conclusion that genetic specificity may reside primarily in DNA has become widely accepted. The identification of DNA as the active transforming principle of the pneumococcus (20) and the elucidation of its role in heredity in bacteriophages (179) are perhaps the main foundations supporting this conclusion. The absence of DNA from tobacco mosaic and other plant viruses has long indicated that DNA may not be the only genetically competent material. In this connection, the finding that influenza virus contains little or no DNA (3) is of interest, since both mutation and genetic recombination have been demonstrated in this virus.

The structure proposed for DNA by Watson & Crick (435) has important genetic implications (431) and, along with the conclusion of a major genetic function for DNA, has given new impetus to investigations of the nucleic acids. A symposium devoted to the structure and function of the nucleic acids has been published in part (316).

*DNA structure and function.*—Todd (419) briefly discusses the data on which present views of the chemical structure of both DNA and RNA are based and concludes that, although there are a number of aspects in need of further clarification, the broad outline of the chemical structure of these im-



portant substances is now quite well-established. From T2 bacteriophage, a nucleotide with a glucose side chain has been isolated (387), and also a nucleotide of hydroxymethylcytosine (436). Tracer studies indicate that host cell cytosine is the major precursor of the hydroxymethylcytosine of T6, and that the hydroxymethyl group may be derived from the  $\beta$ -carbon of serine (85). A new purine constituent of DNA has been found following mild hydrolysis (246). It has not been found in RNA.

Crick (90) briefly discusses the experimental evidence underlying the proposed complementary helical structure of DNA (435). One of the important features of this structure is the ease with which the exact replication of DNA can be explained by the assumption that each member of the two stranded helix separates from its partner and serves as the template for the formation of a new complement. The principal difficulty is the question of how the two helically-wound, polynucleotide chains are separated. Delbrück (99) has elaborated a mechanism which solves this difficulty and at the same time has a number of implications which strengthen its plausibility. Delbrück suggests that the synthesis of new complementary chains on each of the complementary chains of the parental structure proceeds synchronously along the two chains and that, as the synthesis proceeds, the chain breaks at every half-turn of the helix. The lower terminals of the breaks are immediately rejoined to the open ends of equal polarity of the new chains. Two other mechanisms have been suggested which would permit separation of the helically-wound, polynucleotide chains without breakage (149, 322).

Dekker & Schachman (97) consider at some length various models of DNA structure in the light of the available physical and chemical data. They propose a modification of the Watson-Crick model in which the two strands are interrupted about every 50 nucleotides, the breaks in the two chains being staggered relative to one another.

Successive extraction of deoxynucleoproteins with increasing molarity of NaCl yields fractions in which the ratio of adenine and thymine to guanine and cytosine varies significantly (89). Lucy & Butler (254) showed that a progressive increase in this ratio could be obtained by successive extractions with the same molarity of NaCl. In both of these studies, the molar ratios of adenine to thymine and guanine to cytosine were not significantly different from unity, in conformity with the complementary helical model.

The ionic environment, the pH, and various nonionic compounds have a significant effect on the ultraviolet absorption spectrum of sodium deoxyribonucleate (49). Frick & Rosenberg (142) interpret the changes in ultraviolet absorption induced by pH as due to a breaking of intramolecular hydrogen bonds, basing their conclusion on differences in infrared absorption spectra. A similar conclusion was made by Laland *et al.* (230) for ultrasonically treated DNA. The changes in infrared absorption spectra of DNA following DNAase treatment, heating, or raising the pH over 9 were likewise suggested as the result of hydrogen bond breakage (50).

Volkin & Cohn (427) review the chemical methods for the preparation

and analysis of DNA. Differences in the amount of protein present did not affect the relative quantities of fuchsin and DNA in Feulgen-treated nuclei (385) and a procedure was devised for the quantitative determination of bacterial DNA by means of the Feulgen reaction (102). Some of the common methods of DNA preparation were compared (141).

Bolton (52) has studied a number of compounds which can supply carbon for nucleic acid synthesis in *E. coli* and has examined the utilization of C-14 labeled pyrimidines. From studies using labeled purines and purine derivatives, it is suggested that the purine components of RNA and DNA follow largely similar if not identical synthetic pathways (25). In rapidly growing cultures of *E. coli*, Hershey (178) failed to observe replacement of phosphorus or purine and pyrimidine carbon in DNA, or transfer of these atoms from RNA to DNA. Similar findings are reported by Fujisawa & Sibatani (146). The negligible replacement rate of phosphorus for DNA in growing *E. coli* is somewhat at variance with Delbrück's proposed mechanism for DNA replication (99), which would permit a maximum exchange of 10 per cent per replication cycle.

The interesting observation that certain halogenated pyrimidines can be incorporated into the DNA of *E. coli* and of some of its bacteriophages was reported simultaneously by Dunn & Smith (110) and Zamenhof & Griboff (469, 470). An analogue of thymine, 5-bromouracil, was shown to replace 18 to 35 per cent of the thymine in the DNA of certain strains of *E. coli* grown in media containing this halogenated pyrimidine. Replacement of up to 80 per cent of the thymine in phage T2 DNA by 5-bromouracil was accompanied by an increase to about 70 per cent of noninfective particles (110). On the basis of a random distribution of 5-bromouracil among the phage particles, it seems likely that particles containing the halogenated pyrimidine are infective but that the probability of successful infection decreases with increasing incorporation of the thymine analogue. Careful controls were included in the experiments (469). No appreciable incorporation of halogenated pyrimidines into RNA was observed (110). No mass genetic effects were observed (470). Zamenhof (468) failed to observe incorporation of 5-bromouracil in the DNA of *Hemophilus influenzae* under conditions in which 5 per cent of the thymine of *E. coli* DNA was replaced.

The effect of various physical and chemical factors on the DNA of the ox and the human has been compared with their influence on the transforming activity of *H. influenzae* (471). A correlation was observed between the carcinostatic efficacy of a series of nitrogen mustards and their power to inactivate the transforming DNA of *H. influenzae* (473). Heat and various mutagenic treatments were shown to produce a labilization of DNA with transforming activity (472). Attempts to isolate a deoxyribonucleoprotein from the pneumococcus strains producing active transforming principle were unsuccessful (119).

Treatment with DNAase impairs or destroys the ability of isolated calf

thymus nuclei to incorporate  $C^{14}$ -alanine into nuclear protein; this activity was restored by addition of DNA to the enzyme-treated nuclei (9). An important contribution to the knowledge of the functions of nucleic acids was made by Gale & Folkes (147) who demonstrated the role, previously only inferred, of both DNA and RNA in protein synthesis. Significant increases in protein nitrogen, in the enzymes forming acids from glucose, in catalase, and in the inducible enzyme  $\beta$ -galactosidase were observed upon the addition of DNA or RNA to staphylococcal cell fragments from which most of the DNA and RNA had been removed. A similar stimulation, resulting from the addition of a purine-pyrimidine mixture, indicates a synthesis of nucleic acids followed by protein synthesis. Gale & Folkes briefly discuss possible mechanisms.

Gamow (148) puts forward an interesting suggestion concerning the possible relation between DNA and protein structures. Noting that the DNA molecule is composed of parallel chains consisting of only four different kinds of polynucleotides whereas proteins consist of peptide chains formed by about 20 kinds of amino acids, Gamow suggests that the translation from a four-digital to a twenty-digital system may be the result of a "key and lock" relationship between the various amino acids and the rhomb-shaped "holes" formed by the nucleotides in the DNA chain. There are 20 different possible types of such "holes." They could comprise the template for the synthesis of peptide chains. Delbrück (98) reviews briefly the significance of DNA in phage genetics and considers the structure and duplication of the DNA molecule with emphasis on the transmission of genetic information.

A quite different view of the biological function of DNA is discussed by Marshak & Marshak (267) in an extension of the hypothesis that RNA is the primary genetic material and that DNA functions only in a regulatory capacity.

*RNA structure and function.*—The chemical structure of RNA is discussed by Todd (419). A method of determining nucleotide sequence in polyribonucleotides has been successfully applied to di- and trinucleotides (446). Tobacco mosaic virus and potato virus X contain two types of RNA differing in the end groups (266) and there are indications of the presence of RNA with other combinations of the four possible types of end groups.

When precautions were taken to insure quantitative recovery of the RNA as mononucleotides, Elson & Chargaff (118) observed regularities in the molar ratios of the purine and pyrimidines of RNA from a number of sources. The molar ratios of adenine to uracil, guanine to cytosine, and purines to pyrimidines were generally close to unity; but significant deviations were observed in RNA from *Serratia marcescens* and *E. coli*. The implications of the observed regularities for the structure of RNA were discussed. Similar x-ray diffraction pictures of RNA from a variety of sources were obtained by Rich & Watson (347, 348) who discuss the similarities between RNA and DNA. The x-ray pattern suggests a DNA-like structure for RNA

but the lack of complementary base ratios presents difficulties. If the regularities in composition observed by Elson & Chargaff (118) prove to be typical, a structure for RNA resembling that for DNA would appear possible.

The effect of RNA on protein synthesis by staphylococcal cell fragments (147) has been discussed previously. Additional indications of the relation between RNA and protein synthesis are supplied by the high correlation between RNA content and net protein synthesis of a number of *Neurospora* mutants (404), and by the inhibition of amino acid incorporation into the protein by RNAase in onion root tip cells (56). Roth (356) makes the interesting suggestion that the function of intracellular RNAases may be to free newly synthesized protein from the postulated RNA template by the enzymatic degradation of the template. The role of the nucleus in maintenance of the RNA content of *Amoeba proteus* has been demonstrated (214).

Mutation phenomena and the physical and chemical structure of tobacco mosaic virus have been reviewed by Schramm (378). A helical structure for tobacco mosaic virus nucleoprotein is proposed by Watson (434) on the basis of x-ray diffraction evidence. Groups of tobacco mosaic virus strains which differ sharply in the symptoms produced in *Nicotiana tabacum*, in serology, and in various physical and chemical properties, also vary in the ability to form a stable complex with a nucleoprotein derived from the host cells (155). The formation of the complex is responsible for the difference in color of the virus protein resulting from different methods of purification. Wang & Commoner (430) have isolated and analyzed an infectious ribonucleoprotein formed in addition to tobacco mosaic virus in leaves infected with the latter. Significant differences in amino acid composition, isoelectric point, and electrophoretic mobility at different pH values were observed. The nucleoprotein is similar to tobacco mosaic virus in size and shape, serology, RNA content, and infectivity. It is felt that the nucleoprotein represents an intermediate or alternative product in the biosynthesis of tobacco mosaic virus.

#### SPONTANEOUS AND INDUCED CHANGE OF GENETIC MATERIAL

Two volumes containing extensive reviews of the biological effects of both high and low energy radiation with emphasis on the genetic and cytogenic effects have been published (192).

*Inactivation.*—A number of papers have been concerned with the kinetics of inactivation and its significance. Sacher (364) concludes that the survival curves of *E. coli* B and B/r are composed of two or more components and that the extreme sensitivity of B is due to presence of a particular component absent from B/r. Both survival curves are characterized by a final exponential branch with a slope that is invariant in both strains under all pre- and post-treatments studied.

Survival curves of *E. coli* have also been analyzed by Houtermans (200, 201) who concludes that exponential curves are obtained only from widely variable cultures and that survival curves can be interpreted largely on hypotheses other than the target theory. Somewhat similar views of the multi-

ple nature of ultraviolet injury have been expounded in studies of metabolic reactivation (175, 177). Stein (399), however, suggests an interpretation similar to the target theory for the inactivation of *E. coli* with acridine orange.

Some interesting work using low-voltage electrons has permitted the identification of three zones of sensitivity in *Bacillus subtilis* spores. No radiological evidence of a discrete organized nucleus was obtained (92).

Rubin (360) has shown that growing *E. coli* B and B/r cells can withstand continuous radiation at dose rates of up to 10,000 r per hour with little effect on growth rate, and offers the interpretation that growing cells form a substance which counteracts the toxic effects of substances produced by radiation. These observations are in sharp contrast to the greater radiosensitivity observed for growing cells in higher organisms.

Powers (332) discusses the effect of postirradiation temperature upon cell death, death after autogamy, and suppression of cell division in *Paramecium aurelia*. Induction of lethal mutation, as indicated by death after autogamy, is independent of postirradiation temperature; but the rate of recovery from the suppression of cell division increases at higher temperatures. Equations for fitting a logistic function to the recovery from suppression of cell division are developed and their significance discussed. The relative biological activities of hydrolytic products of nitrogen mustard have been measured in *P. aurelia* (334). Indirect evidence has been obtained which indicates that postautogamous death of *P. aurelia* after irradiation or aging cannot be attributed entirely to lethal mutations (276).

For the inactivation of haploid yeast, 3.2 Mev  $\alpha$ -particles showed a maximal effectiveness twice that of 220 kvp x-rays; densely ionizing, 120-Mev carbon nuclei showed the same effectiveness as 220 kvp x-rays (374). An interesting interaction between starvation and the relative effectiveness of 3.2 Mev  $\alpha$ -particles and 50 kvp x-rays for inactivation of haploid yeast cells was observed;  $\alpha$ -particles were less effective than x-rays in killing fresh cells, but more effective in killing starved cells (117).

Deuterons,  $\alpha$ -particles, and electron bombardment of T1 bacteriophage decreased serological affinity (324) and increased the latent period (323). Davis (93) has obtained some interesting results on the inactivation of T1 phage with 100-2000 volt electrons with penetrating ranges of 10 to about 1100 Å. Evidence of internal differentiation was obtained, and it was concluded that the inactivation of T1 could be achieved by inactivation of a single surface unit with a molecular weight of about 50,000.

A detailed analysis has been made of the kinetics of inactivation of T1 phage when exposed to ultraviolet under different physical and chemical conditions (185). Action spectra for inactivation of T1 and T2 phages with maxima at 2650 Å were obtained; the quantum yields for inactivation were constant between 2250 and 3022 Å (474). Using methylene blue as the photosensitizing dye Welsh & Adams (442) failed to observe photoreactivation or multiplicity reactivation of photodynamically inactivated bacteriophages.

The effect of ultraviolet irradiation on the toxicity, hemolytic activity, respiration, and infectivity of rickettsiae has been studied (8).

*Factors influencing inactivation.*—(a) *Oxidation state*: Exponential survival curves are obtained for haploid yeast cells exposed to x-rays under either anaerobic or aerobic conditions. The critical factor is the concentration of oxygen during the actual exposure; anaerobic growth prior to exposure had no additional effect (47). A large variety of carbon substrates and growth conditions were without appreciable influence on the x-ray sensitivity of *E. coli* B (162). Alper (11) has shown that surviving S-13 bacteriophage particles are "part-inactivated" by exposure to x-rays. Such particles are very sensitive to reducing radicals and to  $H_2O_2$  acting as a reductant after decomposing into active radicals under irradiation (10). In contradistinction to most biological systems, indirect x-ray inactivation of S-13 phage is due to reducing radicals and the presence of oxygen protects the phage against the indirect x-ray effects. A detailed series of reactions is postulated (112) and a predicted pH effect was realized in experiment with lower pH values which favor  $H_2O_2$  production resulting in greater survival (12). A similar protection of T1 phage against x-ray inactivation by oxygen was observed (21).

(b) *Temperature and phase state*: With the general conclusion that about half the effects of x-rays on cells in oxygenated suspensions is due to indirect mechanisms involving radio-decomposition products of water, it would be expected that temperature during irradiation would have a small effect on inactivation rate. Furthermore, a large increase in effectiveness would be expected with the change from the solid to the liquid state which permits much greater diffusion of radiation-produced radicals. These expectations have been confirmed by a number of investigations which are in close agreement. These studies have involved x-ray inactivation of bacteria (203, 396), of yeast (459), of bacteriophages (460), and  $\alpha$ -particle effects on *E. coli* (203). A similar effect of temperature was noted in a phage labeled with  $P^{32}$ . The significantly lower inactivation at  $-79^\circ C$ . as compared to  $-2^\circ C$ . suggests that  $P^{32}$  decay to S contributes only slightly to inactivation (74).

(c) *Miscellaneous chemical and physical factors*: Bachofer & Pottinger (22) have shown that various inorganic ions produce a significant protection against inactivation of T1 phage by x-rays. The protective effect of the various salts against heat inactivation was in inverse order to the x-ray protection (23). The mechanisms involved in the protection are not completely known. The cyanide ion also possesses a significant protective effect for x-rayed T1 (24). Hydrostatic pressures up to 10,000 lb./sq. in. were without effect on the x-ray inactivation of haploid yeast cells, which suggests that macromolecular unfolding is not initially involved in the killing (70).

(d) *Nuclear constitution*: Complex variations in the sensitivity of budding yeast cells to x-rays (372) and ultraviolet (371) were observed. Both the sensitivity and the form of survival curves vary. A careful study (36) of the radiation resistance of haploid yeast cells revealed that the observed survival curves reflected two components: 90 per cent of the cells are sensitive



with an LD50 of 3100 r and are inactivated exponentially; the remaining 10 per cent yield a sigmoidal survival curve, and are very much more resistant, having an LD50 of about 60,000 r. This resistant component is composed of budding cells, as shown by microscopic examination and by the production of homogeneous nonbudding populations through starvation. Addition of nutrients to such starved populations results in significant synchronous division and permits demonstration of the resistant components. Ultraviolet inactivation does not reveal a similar resistant component: the inactivation curves are sigmoidal, and are not simplified by starvation (36).

Somewhat similar changes in  $\alpha$ -particle and ultraviolet sensitivity of *E. coli* at different growth stages were observed (202). In general, the cells were most resistant just before division and most sensitive just after division.

The induction of recessive lethal mutations in diploid yeast cells by x-rays was indicated by reduced spore viability in asci from irradiated stocks (284). In the same study, triploids and tetraploids were found to be intermediate in x-ray sensitivity between haploids and diploids, suggesting the presence of dominant lethals. A large cell strain of *E. coli* exhibits ultraviolet inactivation kinetics compatible with a possible polyploid constitution (475).

Norman (299) has extended his studies indicating that ultraviolet inactivation of *Neurospora* conidia is due to nuclear inactivation. The inactivation cross section per nucleus in uninucleate conidia is about twice that in multinucleate conidia. Photoreactivation of one nucleus appears sufficient for photoreactivation of a conidium.

**Reactivation and Recovery Phenomena.**—The inhibition of budding in a polyploid series of yeast by ultraviolet has been shown to be photoreversible (370). After photoreactivation, exponential curves were observed for all members of the polyploid series whereas sigmoidal curves result from 2n, 3n, and 4n yeast exposed only to ultraviolet.

When phage-bacterium complexes formed by single infection with T2 or T7 are exposed to ultraviolet, then to maximal photoreactivating light, and again to ultraviolet, the final sensitivity is the same as the initial sensitivity (241). This observation indicates that photoreactivation is effected by a direct reversal of ultraviolet damage. Hill & Rossi (184) have studied the influence of a variety of conditions of irradiation on the ultraviolet sensitivity and photoreactivation of T1 phage. The failure to observe photoreactivation after ultraviolet irradiation of dried phage is not due to an impairment of adsorption (186). A number of models for the ultraviolet inactivation of T1 phage are discussed in the light of the survival curves obtained (185).

Starvation of *Colpidium colpoda* prior to irradiation increases sensitivity to ultraviolet and reduces sensitivity to photoreactivating light (152). Prior exposure of the starved animals to blue light and irradiation in  $10^{-4}$  M glutathione resulted in some protection against ultraviolet damage. Ducoff (107) has investigated the nutrient requirements for recovery from the radiation-produced inhibition of cell division in *Chilomonas paramecium* and in *Tetrahymena pyriformis*.

Hollaender & Stapleton (193) briefly summarize the effect of postirradiation conditions on the recovery of x-rayed *E. coli*. The influence of post-irradiation incubation temperature is emphasized and a pronounced effect of yeast extract in stimulating recovery at 25°C. is shown. Investigations of metabolic reactivation have shown that ultraviolet produces a number of metabolic disturbances (175) and that substrates belonging to the citric acid cycle were most effective as reactivating agents (177). Most enzyme inhibitors were shown to inhibit metabolic reactivation (176).

Work on reactivation by catalase following ultraviolet irradiation is briefly summarized (236). One of the interesting features is the relation of reactivation by catalase to lysogeny. Miletic (272) also points out that appreciable reactivation by catalase is observed only in lysogenic strains and is manifested both by an increase in the number of surviving bacteria and by an increase in the number of surviving productive complexes which have been induced by the radiation. The restoration of productive complexes by catalase was observed following irradiation with both ultraviolet and x-rays (272). Not all lysogenic strains exhibit catalase restoration. It does not occur in the Lisbonne strain of *E. coli* which, interestingly, is not inducible by ultraviolet (417). Nonlysogenic strains of *E. coli* K-12 were also observed to exhibit catalase restoration (236, 417).

*Physiological effects of radiation.*—The dose-effect curve for the x-ray retardation of budding in yeast has two exponential components, one of which is integrally related to the order of polyploidy (373). Cell division in bacteria and yeast was shown to be selectively inhibited by x-rays with only a slight effect on growth (393). Errera (123) has studied the ultraviolet induction of filaments in *E. coli* B. Cytoplasmic synthesis, growth and nuclear division continue but a block in cell division results from the radiation. Hydrosulfite, adenosinetriphosphate, and visible light were found partially to reverse the block in cell division. A thymine-requiring mutant of *E. coli* was found to be capable of cytoplasmic synthesis in the absence of appreciable DNA synthesis (83). Continued unbalanced cytoplasmic growth leads to the irreversible loss of the ability to divide and the cell dies. Cohen & Barner (83) suggest that death from ultraviolet may also be the result of such unbalanced growth, as indicated by filament formation and by the specific inhibition of DNA synthesis with ultraviolet doses that produce much less effect on RNA synthesis and cytoplasmic growth (218).

Persistent, slow growing clones of *Chilomonas paramecium* have been found after x-radiation (106). Their high incidence and delayed appearance indicate that they are not due to gene mutations. Exposure to 80,000 r of x-rays had no effect on aging in *Paramecium aurelia* (222), indicating that accumulated macronuclear mutations are probably not the cause of the decline in division rate observed in the absence of nuclear reorganization. In addition to inhibiting the mean division rate, x-rays were also observed to increase the variability of interdivision times and growth rates of *P. aurelia* (365).



Billen & Volkin (44) observed differences in the macromolecular constituents of cell free extracts of x-rayed *E. coli*. No differences were observed unless the irradiated cells were incubated for a time in the presence of an energy substrate.

*Mutation.* An unusual mutation-dose curve has been observed with morphological mutations of *Streptomyces* induced by x-rays (292). Extremely high proportions of mutants are observed and, although the dose-effect curve is initially linear, a saturation level is reached. Similar nonlinear or saturation effects are observed for both the photoreactivable and photo-stable ultraviolet mutagenic effects and, although the initial steps are different, a common mechanism responsible for the saturation is postulated for both x-rays and ultraviolet (292). A linear mutation dose-curve is reported for x-ray induced auxotrophic mutations at a glutamic acid locus in *Neurospora* (462). A differential response to different mutagens was observed at an ornithine locus in *Neurospora*; the conditions under which conidia were exposed to ultraviolet also produced a differential mutational response (461).

Rubin (360) observed that the induced mutation rate to streptomycin resistance in continuously x-rayed, growing *E. coli* cells was independent of the growth rate and, just as for resting cells, depended only on the total x-ray dose. Induced mutations, therefore, do not appear to result from errors in gene duplication.

A well-substantiated case of a mutator gene is reported in an *E. coli* K-12 strain (421). The mutator strain exhibits mutation rates to streptomycin resistance about 100 times those in non-mutator strains, coupled with a remarkably low proportion of streptomycin dependent mutants. Mutation rates at other loci are also influenced and the mutator factor apparently segregates in the usual fashion in crosses (421).

Atwood & Mukai (18) report observations on survival and lethal mutations of *Neurospora* conidia exposed to a nuclear detonation. The report is valuable for two reasons: Firstly, no effect of intensity of neutrons on survival was observed even though calculations indicate that an intensity of about 10 billion r per second was reached. Secondly, it is the most extensive published description of some of the very critical heterocaryon methods they have developed for the analysis of lethal radiation effects and the most extensive demonstration of the usefulness of the methods.

A number of variables influencing the indirect mutagenic action of ultraviolet in bacteria have been investigated. Factors studied included the genetic locus, the nature of the substrate irradiated and its concentration, pH, and temperature (451). It was concluded that the amount of  $H_2O_2$  produced and its stability were two of the major determinants of the genetic effect obtained. Kimball (221), however, was unable to demonstrate a mutagenic effect of either added or radiation-produced  $H_2O_2$  in paramecia whose catalase was inhibited by cyanide. He concluded that  $H_2O_2$  cannot be a major factor in the production of mutation by x-rays in paramecia.

A series of carcinogens was shown to be mutagenic for *E. coli* (377). The

mutagenicity appeared to be correlated with carcinogenicity when the solubilities of the compounds were considered. Demerec *et al.* (100) have screened a number of compounds for mutagenicity in various *E. coli* strains in order to determine whether a correlation exists between their mutagenicity and their effectiveness in cancer chemotherapy. The different specific mutation systems employed react differentially to certain of the compounds. These same workers have observed a number of mutations in *E. coli* which are specifically stable to mutagens (100).

Irradiation of the sensitive host cells before infection with  $\lambda$  phage resulted in an absolute increase in the number of virulent  $\lambda$  mutants (209). Ultraviolet irradiation of T2-infected cells is reported to increase the frequency of host-range mutants (235). Murphy (285) has isolated at least 18 types of plaque mutants which are produced spontaneously at quite high rates by a lysogenic *B. megaterium* strain. The overall mutant frequency is about 1/2000; individual frequencies of the different mutant types range from 1/7000 to  $10^{-5}$ .

Ryan and his co-workers (362, 363) have focused attention on the segregation lag in expression of mutations in multinucleate bacteria and have emphasized that accurate estimates of mutation rates must include consideration of the average number of nuclei or mutable units per cell. Growing cells of a histidineless *E. coli* contain four nuclei, and mutation to histidine independence in such cells produces a heterocaryon in which the histidine independent nucleus is dominant; the histidine independent phenotype is expressed after a short delay lasting less than one generation (362). Ryan (361) demonstrates that a variable delay in onset of growth is another important factor in the delayed appearance of induced mutants. The length of the delay after large doses of ultraviolet was too long to be accounted for by nuclear segregation and phenotypic delay. Ryan suggests that the mutations occurred in a fraction of the cells receiving an injury which delayed the onset growth and at the same time made mutation likely. Witkin (456) has shown that the delay in appearance of *Salmonella* prototrophs produced by transduction is less than in ultraviolet induced prototrophs.

## METHODS

Reference has been made to the induction of synchronous division of bacteria (198, 233) and of *Tetrahymena* (376). Synchrony is produced by temperature changes and the method has been successfully applied to study transformation phenomena and lysogenization in bacteria.

Two modifications of the chemostat for maintaining bacterial cultures in a steady state have been devised (227, 300). Micromanipulative methods have been developed for single cell isolation of lipophilic and microaerophilic bacteria (122) and the oil chamber has been adapted for routine isolation of single bacterial cells (239).

A filtration technique for isolation of biochemical mutants of *Neurospora*

depends on the removal of nonmutant mycelia by filtration after incubation in a medium which does not permit growth of mutant conidia (75, 463).

An accurate layer-plate technique for assaying rates of mutation to streptomycin resistance has been developed (359). Inoculation of a series of flasks with inocula of variable size is used to estimate mutation rates by calculations based on the inoculum size and frequency of sub-cultures containing no mutants (335). Membrane filters have been successfully employed in estimations of the rate of mutation to streptomycin resistance (268). A simple procedure involving the growth of bacteria on filter paper strips placed on agar surfaces permits the phenotypic expression of induced mutations without time-consuming centrifugations (111).

A simpler technique has been devised for obtaining mating types in heterothallic yeasts (449). Replica plating has been utilized in determination of carbon assimilation patterns in yeasts (384). The inability of respiration-deficient yeasts to produce excess alkali during growth on a medium containing sodium acetate is the basis for an indicator medium useful in screening for respiration-deficient strains (304).

An ascomycete with a spore color mutation permits direct observation of segregation of the ascus (48). Atwood & Mukai briefly describe some of the techniques employed in large scale studies of lethal mutations in *Neurospora* (18, 19).

Studies of intracellular phage growth are facilitated by premature lysis of infected cells with penicillin (31). Dulbecco & Vogt (108) have extended the invaluable plaque-formation technique to poliomyelitis virus strains. Modifications of the technique for deembryonating eggs and methods for study of recombination in mixed infections of influenza virus are presented (68).

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# GROWTH OF BACTERIA<sup>1</sup>

By AARON NOVICK

*Committee on Biophysics and Department of Microbiology,  
The University of Chicago, Chicago, Illinois*

The general laws of bacterial growth have been known for many years. Modern concepts were discussed by Monod (1) in his excellent review of 1949. There he clearly pointed out, for example, that the size of a bacterial population after a certain time will depend on three constants: the growth lag, the growth rate, and the ultimate population that can be supported by the medium.

In recent years there has been a revival of interest in the general patterns of microbial growth. One principal reason has been the development of continuous culturing techniques for microorganisms and of the theory underlying such methods. This development will be the principal subject of this review. In order to clarify subsequent discussion, a brief review of the theory underlying continuous flow systems will be given before attention is turned to a description of the development and application of such systems.

The second and more recent reason for renewal of interest in microbial growth is the discovery that considerable synchronization of divisions in large populations can be achieved by periodic changes in the incubation temperature. A brief discussion of these experiments follows immediately.

## SYNCHRONIZATION EXPERIMENTS

Zeuthen & Scherbaum (2, 3) have reported the successful induction of synchronized cellular divisions in mass cultures of the protozoan *Tetrahymena piriformis*. Alternate periods of incubation of one-half hour at 29° and one-half hour at 33° during an elapsed time of six to ten hours are followed by 85 per cent synchronization of the first, 80 per cent of the second, and 65 per cent of the third division following the cessation of treatment.

Hotchkiss (4) has described experiments with a strain of pneumococcus in which organisms growing at 37°C. were incubated for 15 min. at 25°C. and then returned to 37°C. Upon further incubation at 37°C., transformability to streptomycin resistance by the DNA of a streptomycin-resistant organism showed a striking periodic fall and rise. Examination of the viable cell count showed a similar periodicity, with one-half of the total cell divisions occurring within the last 5 min. of a thirty-minute cycle. Correlation of this periodicity in terms of growth phase of the bacteria and transformability is made difficult by the fact that the divisions seem to occur with a period of 30 to 35 min. while transformability occurs with a 40 min. period. It should

<sup>1</sup> The survey of literature pertaining to this review was completed in January, 1955.



be noted, however, that synchronization persists for at least two generations after the initial 25° treatment.

Maaløe & Lark (5, 6), working with *Salmonella typhimurium*, report that they were able to induce nuclear as well as cellular divisions with temperature shifts. They found that, at low bacterial densities, waves of bacterial division are obtained when the bacteria are incubated successively for 30 min. at 25° and 6 min. at 37°; with higher densities a regime of 28 min. at 25° and 8 min. at 37° is successful. After several such cycles, 80 to 90 per cent of the bacteria divide during the 6 min. they are kept at 37°. Cytological studies show that nuclear division occurs 4 to 8 min. after a shift from 25° to 37°, and it was also observed that the fraction of bacteria becoming lysogenic when aliquots are treated with a temperate phage doubles at the same time. In this system, as distinct from the two cases described earlier, continued synchronization requires the continuation of the alternate temperature changes. A temperature cycle has also been used to synchronize divisions in a population of the ameba *A. proteus* (55).

#### THEORY OF CONTINUOUS FLOW SYSTEMS

The theory has been given by Novick & Szilard (7, 8) and by Monod (9) and is briefly summarized here. A very similar analysis has been developed for continuous systems designed to study rates of chemical reactions and rates of crystallization by Denbigh (10) and by Bransom, Dunning & Millard (11), respectively.

A continuous culturing device in general consists of a reservoir of sterile liquid nutrient, a feeding system that admits this nutrient liquid at a constant rate to a growth tube in which the bacteria grow, and an overflow through which the bacterial suspension leaves the growth tube at a rate that maintains the volume of culture in the growth tube constant. In addition, provision is made for the mixing of the culture and aeration when needed. The flow of liquid from the reservoir into the growth tube, and the resulting departure of the bacterial suspension from the growth tube, will be assumed to occur at a rate of  $w$  ml./hr, and the growth tube to have a volume of  $V$  ml. Now if the law for the growth of bacteria in the usual undiluted system be written as

$$\frac{dN}{dt} = \alpha N \quad 1.$$

where  $N$  is the number of bacteria per ml.,  $t$  the time and  $\alpha$  the mean division rate,<sup>2</sup> the growth law in a continuously diluted system of the kind described above becomes

$$\frac{dN}{dt} = \alpha N - \frac{w}{V} N = \left( \alpha - \frac{w}{V} \right) N \quad 2.$$

<sup>2</sup>  $\alpha$  is related to the doubling time ( $t_{1/2}$ ) by the expression

$$\alpha = \frac{\ln 2}{t_{1/2}}$$

since the bacteria have grown by the fraction  $\alpha$  and have been diluted by the fraction  $w/V$  in unit time.

From equation 2, it is seen that if  $w/V$  is greater than  $\alpha$ , the culture will be diluted out more rapidly than it can grow and will eventually be completely washed out of the growth tube. On the other hand, if  $w/V$  is less than  $\alpha$ , it is evident that the bacteria are then growing more rapidly than they are being washed out and that the density of bacteria in the growth tube will increase. Obviously, if the density of microorganisms is to remain constant,  $w/V$ , the flow rate, must be equal to  $\alpha$ , the growth rate.

Such an equality of growth rate and flow rate can be obtained in two ways. The first method uses some system to observe the density of microorganisms; for example, a light source and photocell, or some device that measures a consequence of change in bacterial number such as pH or a chemical concentration. This density-measuring system is used to control the flow rate. When the flow rate is less than the growth rate, the density,  $N$ , will eventually become larger than the desired level  $N_0$ . When  $N$  becomes larger than  $N_0$  the photocell commands an increase in flow rate. Should the density fall below the desired level  $N_0$  because the flow rate has become greater than the growth rate, the photocell detecting this decrease in density commands a decrease in flow rate. As the control system corrects the flow rate to maintain constant density, there will be variations in flow rate whose magnitude will depend on the sensitivity of the density-detecting system. The mean flow rate, however, will precisely equal the growth rate since the density  $N$  tends, in a properly adjusted machine of this type, neither to consistently increase or decrease.

In any such system the organisms will grow at the maximum rate characteristic of the particular choice of chemical and physical conditions. The density of organisms selected to be constant is arbitrary within the following range: that it be sufficiently large for changes in it to cause detectable changes in optical density, but not larger than the nutritive capacity of the nutrient liquid employed. In such a system then, the experimenter can select the density of microorganisms desired over fairly wide limits, but he can only produce a change in growth rate by a change, for example, in choice of nutrient medium or incubation temperature. He can, however, obtain an accurate measure of the growth rate from the value of the mean washing-out rate.

Another, completely different principle for stabilizing a continuous culture machine has been described by Novick & Szilard (7, 8) (the Chemostat) and by Monod (9) (the Bactogen). This scheme differs from that just described in that the flow rate is held at some fixed value below the maximum growth rate. Furthermore the nutrient medium is composed of a large excess of all required nutrients but one. This nutrient has been called the limiting or controlling growth factor. Under these conditions the bacterial density will increase with time, the growth rate being greater than the washing-out rate. However, as the bacterial density increases, the concentration of the limiting growth factor will tend to become very small. Now the growth rate

of an organism as a function of the concentration of a required nutrilit is in general something like that shown in Fig. 1, where the growth rate of a tryptophan-requiring *E. coli* mutant is given as a function of concentration of tryptophan. At higher concentrations of this factor, the growth rate does not depend on the concentration of the factor. However at much lower concentrations [(~4 mg/l for glucose (12) and 1  $\mu$ g/l for tryptophan (8)] the growth rate does depend on the concentration of this required factor. Hence, in the case under discussion, as the rising microbial density finally lowers the

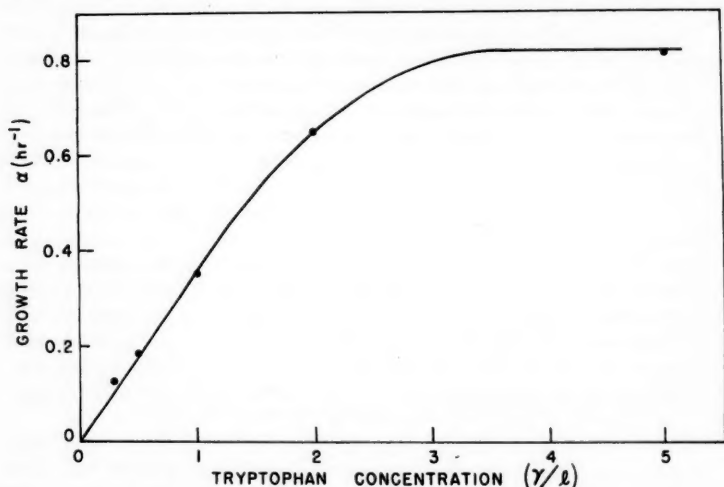


FIG. 1. Growth rate of a tryptophanless strain of *E. coli*, B/1, *t*, as a function of tryptophan concentration.

concentration of the required factor to such a dependent region, the microbial growth rate itself will begin to decrease. And as will be shown below, the growth rate will in fact fall until it becomes equal to the washing-out rate. At this point the system becomes stabilized, the bacterial growth rate now remaining equal to the washing-out rate, and the bacterial density remaining constant at a value  $N$ .

In the present case, equation 2. should be rewritten as

$$\frac{dN}{dt} = \alpha(c)N - \frac{w}{V} \quad 3.$$

where  $\alpha(c)$  indicates that the growth rate  $\alpha$  is now a function of  $c$ , the concentration of the controlling factor in the growth tube. Here initially  $\alpha(c)$  is larger than  $w/V$ , making  $dn/dt$  positive. But as  $N$  increases,  $c$  will fall,  $\alpha(c)$  will fall, and  $dN/dt$  will tend toward zero, leading to a steady state where

$$\alpha(c) = \frac{w}{V} \quad 4.$$

This system is seen to be self-stabilizing, for if  $N$  were for some reason to become too high,  $c$  would fall to a lower value, causing a fall in  $\alpha(c)$  and making  $\alpha(c) - (w/V)$  negative, which would lead to a fall in  $N$  toward the steady state value. That the system is not only self-stabilizing but also free from oscillations due to overshooting has been shown by Spicer (13).

The density  $N$  of microorganisms that will be maintained in the growth tube has been given as

$$N = \frac{a - c}{Q} \cong \frac{a}{Q} \quad 5.$$

where  $a$  is the input concentration of the controlling growth factor,  $c$  the concentration in the growth tube, and  $Q$  the amount of the factor required to make one organism, the assumption being made that  $Q$  is independent of  $c$ , the concentration of the factor at which the organisms take it up (8). In general,  $c$  is very small compared to  $a$ ; and  $N$  can be set equal to  $a/Q$  within experimental error.

Here then is a scheme for the continuous culture of microorganisms that permits not only the arbitrary selection of the bacterial density desired but also a choice of growth rate.

The concentration of the controlling factor,  $c$ , in the steady state in the growth tube of course also remains unchanged. It should be noted in addition that this concentration depends only on the growth rate (or flow rate) selected and is independent of the input concentration of the controlling growth factor. This follows from equation 4, where it is seen that  $\alpha(c)$  and of necessity  $c$  are determined only by  $w/V$ .

A wide variety of substances can be used as the controlling growth factor in such a system. The substance need only be required by the organism and also have a region of concentration where growth rate is concentration dependent. Among the controlling growth factors that have been employed are (a) a required amino acid [tryptophan (8), arginine, proline, histidine (14)], (b) the energy source [lactate (15), glucose (9), maltose (16)], (c) the nitrogen source [ammonia (15)], (d) phosphate (15, 17), and (e) sulfate (17).

There is the restriction in this system of course that the organism used be capable of regulating its growth rate so that it can grow at the desired rate. There are no doubt limits as to how slowly an organism will grow before some mechanism is actuated that causes the organism to go into the lag phase or perhaps even to cease to be viable. In one study with *E. coli* (18) the upper limits of generation time seemed to be close to 15 hr., and attempts to lower the growth rate further forced the bacteria into lag.

It has been suggested that the difference between the two systems described above is that in the Chemostat or the Bactogen one is dealing with starved bacteria, while in the photocell controlled apparatus the bacteria are not starved (19). However, it has been pointed out that in the case of

so-called "unlimited" growth the growth rate is in fact limited by some unknown factor; a great advantage of the Chemostat or Bactogen is that they provide experimental systems in which the factor limiting the growth rate not only is known but the concentration of which can be varied in a known way (15, 20).

Perhaps the most useful way of distinguishing between these two systems of continuous flow is in terms of the different kinds of limitations on the growth rate, for in each the growth rate is in fact limited. When the growth rate is not dependent on the concentration of some growth factor in the medium, the growth rate must then be limited by the rate of some process occurring within the cell. Hence, one can imagine bacteria growing in a photocell-regulated system as being internally limited and in a Chemostat or Bactogen as being externally limited, the growth rate here depending on the concentration of an externally supplied factor. In fact, to identify this difference serves to illustrate the principles underlying the two systems. The case where the flow rate is determined by the growth rate of the organisms will be referred to as the case of internal control. The case where growth rate is determined by the low concentration of a growth factor will be called external control.

#### DEVELOPMENT AND DESIGN

There are many reports in the literature of devices designed for the continued culturing of microorganisms (7 to 9, 21 to 38). Most of the earlier devices and some of the more recent ones were constructed and operated without an understanding of what it was that maintained the population size constant or of what determined the growth rate.

Among the early workers, Rogers & Whittier (23) realized that in their continuous flow system the size of the population was determined by the quantity of lactose in the incoming medium. They grew *E. coli* and a strain of streptococci at very long washing-out times—24 to 30 hr. A similar system was described by Cleary, Beard, & Clifton (24) for growing *E. coli*, and it was understood here, too, that the population density was determined by the input nutrient concentration.

Ketchum, Bostwick & Redfield (25) pointed out that the population can be limited not only by the available nutrient concentration but also by light intensity for the case of a photosynthetic organism and by the formation of inhibitory products. (It should be noted, however, that the formation of an inhibitory product will indeed limit the size of a population but will not necessarily serve to give a non-oscillating steady state. This will depend on the kinetics of formation of the inhibitor and its manner of action.) Ketchum, Bostwick & Redfield grew a marine diatom, *Nitzschia closterium*, in their continuous system and varied the growth rate by varying the flow rate. Since the nutrient was in excess, either the light intensity or the carbon-dioxide concentration limited the growth rate.

Gerhardt (30) used a continuous system for the culturing of *Brucella suis*.

He employed washing-out times of 3.5 to 8 hr. The minimum generation time for this organism under these conditions is 2.6 hr. Apparently no selection was made of a limiting growth factor, the population density being controlled by some unknown limiting character of the nutrient.

In recent years there have been described a number of continuous culture devices operating according to the principle of internal control. All of these have used photo-cell arrangements to observe the rate of increase of optical density and so to control the washing-out rate. In most cases the volume of culture in the growth tube is kept constant by means of an overflow.

Myers (28) and his group designed in 1944 a photocell-controlled continuous flow apparatus for the culturing of unicellular algae. Two barrier-type photocells were used, one to measure the light transmitted through the culture, the other to control changes in the intensity of the light source. The difference in output of the two tubes is fed to a galvanometer-relay which in turn operates a solenoid valve, which regulates the flow of nutrient from the reservoir into the growth tube. The system is balanced at an optical transmission of 80 per cent, the flow being turned on when the transmission is less than 80 per cent. These workers reported sufficient stability and sensitivity to hold the density of an algal suspension constant to 1 per cent for several days. They employed a growth tube of such size that it contained about 5 mg. dry weight of algae, and reported successful cultivation of *Euglena*, *Chlorella* (several different species), and two species of blue-green algae (39, 40).

Bryson (34), in employing the photometric scheme, has described a similar kind of apparatus, again using a pair of photo-voltaic cells to observe the optical density, and a galvanometer-relay and solenoid-operated hose-cock to regulate the feed. Bryson, moreover, modified his system to include the possibility of the addition to the growth tube of a toxic agent along with the nutrient liquid. This toxic agent was maintained in a series of tubes, each connected with its neighbor by means of a siphon, the concentration in succeeding tubes being increased by a constant factor. This arrangement furnishes an exponential rise in concentration of a toxic agent in the growth tube with time, and is meant to be used for the selection of mutants resistant to toxic agents.

Anderson (36) has constructed an apparatus, which he calls the Auxanometer. This device again includes a pair of photocells to control the action of a galvanometer-relay which operates a magnetic valve, which in turn controls the flow. Aeration is by means of a bubbler so designed that the bubbles do not wander into the light beam and yet produce adequate stirring of the culture. In addition, Anderson employs an automatic recorder to maintain a continuous record of the flow rate by means of a count of the drop rate of nutrient liquid into the growth tube. He reports a slow deposition on the growth tube walls of a light-absorbing film, and to prevent this difficulty he uses a "windshield wiper" arrangement of scrapers made of surgical rubber tubing. He has recently introduced the following additional measures to eliminate the deposition of bacteria on surfaces: (a) A teflon-enclosed iron

rod armature which is held against the inner wall of the growth tube by an external Alnico magnet. As the tube rotates, the scraper is rotated by frictional gearing to the wall and is said to be self-cleaning. (b) A row of cylindrical teflon beads, guided but not closely confined by a platinum tee, which scour the bottom of the tube and are self-cleaning (41).

Anderson states that his apparatus is sufficiently sensitive to maintain a culture of *E. coli* at a constant density of about  $10^7$  per ml. Among the sources of error he finds in his system is the dependency of rate measurements on the constancy of drop volume. Inadequate aeration is avoided by increasing the air supply until no dependence on aeration rate is observed. Finally he states that cells adhering at points not reached by the scrapers may contribute to the observed growth without contributing to the turbidity. This limits his runs to 30 hr., after which time abnormalities due to trapped cells disturb the operation of the system.

Fox (42) has constructed an apparatus called the Breeder which also employs a photoelectric system to control the flow rate. Here again a pair of photocells are used to measure microbial density, but the output is used to operate a microammeter relay rather than the more complex galvanometer photorelay. The glassware and feeding system, except for several modifications, is that described by Novick & Szilard (15). The operation of the Breeder is scheduled by a clock so that every 4 min. the density of the culture in the growth tube is measured. The measurement, lasting 30 sec., includes the following sequence of events: erasure of the last reading, stopping of stirring, turning on the light source, taking the reading, turning off the light, and restarting the stirring. As in the other systems described, a reading higher than a pre-set value causes the feeding system to turn on and a lower reading causes it to stop. The ammeter relay system is set to operate for 5 to 10 per cent changes in an *E. coli* population of  $2 \times 10^8$  per ml. The culture volume is about 25 ml. In these experiments synthetic minimal medium as well as nutrient broth were used.

The principal difficulty found by Fox has been the adherence of bacteria to the walls of the growth tube. These bacteria form a colony, fixed to the tube wall, that is not washed out by the flow system. This colony continuously casts off cells into the liquid. Such a process manifests itself by a "dramatic increase in the growth rate," the number of cells in the volume of the growth tube being increased not only by the reproduction of cells but in addition by the contribution from the walls. To prevent such "wall growth" Fox coated the walls of the growth tube with G.E. Drifilm, added .05 per cent Tween-80, and agitated with a magnetic stirrer. In addition, to overcome difficulties caused by excessive foaming, a platinum filament was maintained at red heat just above the liquid level to destroy any rising foam.

Recently Northrop (38) has described a continuous culture device that is very much like those just described, the principal difference being that a commercial colorimeter is substituted for the photo-cell systems usually employed. He, like Anderson, introduces a series of "windshield wipers" to prevent wall growth.



Despite elaborate measures taken to overcome wall growth, such as those described, prevention for very long periods has not been achieved. Until some way is found to prevent such wall growth indefinitely, internally controlled systems will be able to operate only for restricted periods.

For the design of an apparatus that is operated on the principle of external control a feed system is required whose rate can be maintained constant over long periods of time. Such systems were simultaneously described by Monod [the Bactogen (9)] and Novick & Szilard [the Chemostat (7, 8)]. Monod has constructed two apparatuses: one designed for semimicro use in the laboratory and the other for large scale production of bacteria. The laboratory machine uses for its feed system a serum pump that employs a rotating eccentric drum to continuously squeeze a coiled rubber tube. The growth tube consists of a 2-liter round bottom flask which is turned at high speed on a central axis through the neck of the flask. The culture is kept at a constant volume of 100 to 400 ml. by means of an aspirator tube which sucks liquid out of the growth flask when the volume exceeds the desired value. This leveling tube, the tube supplying the incoming nutrient, a sampling tube, and a tube supplying air, all enter the culture flask through the neck parallel to the axis of rotation. In his large Bactogen, Monod employs a large rotating metal drum that contains a culture volume of 50 l. This machine has been used to make almost one kilogram dry weight of bacteria per day and has been used to grow *B. cereus* as well as *E. coli*.

Novick & Szilard (15) have changed the feed system of their first design, in which a constant flow of nutrient liquid was obtained through the maintenance of a fixed pressure across a capillary resistance, to include a capillary tube that is filled with nutrient liquid to a fixed level by a head of pressure admitted to the reservoir. At short periodic time intervals the liquid in the capillary is ejected into the growth tube. The growth tube volume is kept constant at about 25 ml. by means of a simple siphon, and aeration and mixing are obtained by bubbling air through the growth tube. *E. coli* has been grown at densities up to  $5 \times 10^8$  per ml. and at generation times of 2 to 12 hr.

Monod's small Bactogen differs from the Chemostat in that the Bactogen provides much more effective aeration, thereby permitting the use of much larger populations. The Chemostat, however, is simpler to set up and operate since no moving parts are involved, and it is perhaps less likely to become contaminated than the Bactogen, and has a more accurately defined volume.

Neither Monod nor Novick & Szilard report the difficulties with wall growth found in the internally controlled systems. The explanation probably is that under conditions where the concentration of an externally supplied growth factor limits the growth rate, organisms growing closely together on a wall will soon shield each other from supply of this factor, thus strongly limiting their rate of growth. In internally controlled systems, however, such self-limitation will occur only after a large population has become established on the wall.

Browning & Lockinger (33) have recently described an apparatus inspired by the Chemostat and designed for the culturing of the protozoan

*Tetrahymena geleii*. A constant flow of nutrient liquid is obtained by means of a small rotating motor-driven glass dipper. No information is given as to the nature of the nutrient medium employed nor of the controlling factor, if such was used.

Von Hofsten, Von Hofsten, & Fries (37) have built a modified version of the Bactogen and used it for the growth of the ascomycete *Ophiostoma multi-annulatum* under conditions of glucose limitation.

There are a number of workers currently using flow machines that are more or less minor modifications of the systems described above. In some instances a commercial automatic pipette or a commercial automatic pump has been used for the feed system.

Since in all the apparatuses described above the nutrient liquid enters the growth tube in a dropwise fashion, or by small "shots" from an automatic dispenser, the flow is in principle intermittent rather than continuous. However, since the volumes delivered by each pulse are in general less than  $\frac{1}{2}$  per cent of the growth tube volume, more than 200 additions of nutrient will occur per generation. In this case each addition of nutrient will raise the concentration of the controlling growth factor in the growth tube to a concentration less than  $a/200$ , where  $a$  is the input concentration. It is evident that if  $a/200$  is not too large with respect to  $c$ , the mean steady-state concentration in the growth tube, the assumption of continuity will not be too much in error.

It should also be noted that it is possible to maintain microorganisms growing at a reduced rate by the slow addition of the nutrilitie which is being exhausted in a culture approaching saturation. Normally such a culture passes very quickly through this period of reduced growth rate. But if the required nutrilitie is added at a rate slower than it can be taken up by the organisms, the organisms will lower its concentration until they grow at the rate the nutrilitie is being supplied. Under these conditions, the growth rate must in addition continually decrease since the population sharing this nutrient continually increases.

Jacob (43) has taken advantage of such a scheme to obtain reduced growth rates in *E. coli*. In one case, to a culture in which the ammonia had been exhausted, ammonia was added by the slow hydrolysis of urea by a small amount of urease. In another case, lactose with a small amount of lactase was used to supply glucose to a lactose-negative strain that had exhausted its energy supply. Such schemes for the reduction of growth rate have also been employed to obtain increased production of an antibiotic. For example, Soltero & Johnson (44) added glucose at a slow rate to a culture of *Penicillium chrysogenum* that had exhausted its supply of glucose. They obtained a constant low feeding rate by displacing liquid from a buret by gas that was produced electrolytically with a regulated current.

#### APPLICATIONS

There are a number of advantages of continuous culturing techniques over conventional methods that have made possible a wide variety of appli-

cations. The fact that a growing population is held at constant size over long periods of time is one great advantage over classical methods. A second advantage lies in the fact that the concentration of all chemical substances in the growth tube remains constant. And the feature of the externally controlled system that allows variation in growth rate permits growth rate to become an important experimental parameter.

The ability to maintain a large, constant, growing population for long periods of time offers a method for very precise measurements of bacterial mutation rates (8). Under conditions where mutations occur to a mutant that grows at the same rate as the original organism, there is a linear rise with time in the fraction of such mutants in the population. The slope of the rise gives the mutation rate. Should the mutant grow more slowly, the fraction of mutants will rise to a fixed level that will depend on the relative growth rates and on the mutation rate. If the mutant grows more rapidly, the fraction of mutants rises exponentially.

Novick & Szilard, working with the B strain of *E. coli* or strains derived from it in the Chemostat, studied rates of mutation to phage resistance (8, 15, 45, 46). They found that for mutation to phage T5 resistance no matter whether the ammonia, lactate, phosphate, or an amino acid was made the controlling growth factor, the same number of mutations occurred per hour independently of the growth rate for generation times of 2 hr. to 12 hr. (8, 15). They were able to use this system for the detection of mutagenic activity under conditions where the agent being tested had no appreciable lethal effect (15). The method is sufficiently precise that Novick & Szilard were able to find a group of substances that had an "antimutagenic" effect (45, 46), not only counteracting the effect of certain mutagens but reducing the spontaneous rate to less than one-half as well. They have also been able to demonstrate a series of populational shifts in the Chemostat to bacterial strains able to grow faster at low concentrations of the controlling growth factor (8).

Lee (47), who performed experiments with the same system for the mutation to resistance to phage T6, found the same dependence of mutation rate on absolute time. Fox (42) and Moser (48) have reported that the rate of mutation per hour at short generation times does become dependent on the generation time. De Rothschild (49) studied the mutation of a tryptophan-requiring strain of *E. coli* to tryptophan independence in a glucose-limited Chemostat but found no rise in fraction of mutants with time.

Labrum (50), using a Chemostat, investigated the dependence on generation time of the time required for the expression of induced mutations. Here an attempt was made to inoculate a Chemostat with a number of cells equal to the expected steady-state population. But since these bacteria had just been subjected to treatment with manganous chloride, a very long lag period was anticipated. Labrum attempted to overcome this difficulty by not starting the flow until the end of the lag period. But it is hard to see how this can be done without raising serious questions about the number of generations the bacteria have in fact passed through.

Continuous culture techniques have also proved very useful for the study

of problems of bacterial physiology and biochemistry. The features of constant population size, of constant chemical concentration, and of the ability to vary the growth rate offer many great advantages over older methods. For example, the fact that all chemical concentrations remain constant permits a kind of experimentation otherwise quite impossible. In a flow system, very low concentrations of nutrients can be maintained quite constant despite the fact that they might be taken up at a very high rate. In the externally controlled systems the controlling growth factor is generally present in the growth tube at concentrations that would normally be depleted in a matter of seconds.

Siminovitch & Cohn (51) have studied the dependence of the relative quantities of RNA, DNA, protein, and one enzyme in a bacterial population on generation time and on the nature of the controlling growth factor. In addition, Cohn & Torriani (16) have used a system of external control for the study of the kinetics of formation of a protein related to  $\beta$ -galactosidase. Perret (52) has reported using a similar system to observe the kinetics of penicillinase induction in *B. cereus*. Experiments to measure the rate of protein turnover have been performed in a Chemostat (53).

The externally controlled systems are very useful for the study of the rate of formation of some product of bacterial metabolism. For example, a study was made of a tryptophanless mutant of *E. coli* that poured out into the medium a compound with the same absorption spectrum as tryptophan (14). It was discovered that this compound is made at an appreciable rate only at very low concentrations of tryptophan, and, below a certain level of tryptophan, the rate of synthesis is independent of tryptophan concentration. In another series of experiments it was possible to study the relationship between the concentration of an amino acid in the medium and the rate it is synthesized by a strain capable of such synthesis, despite the fact that it was necessary to maintain constant concentrations of this amino acid as low as one microgram per liter (54).

The applications of continuous culturing devices that have been listed here are only representative of the wide variety of problems uniquely accessible to such techniques. Obviously more widespread application must await more widespread understanding of the principles and increased familiarity with the techniques. On the other hand, more widespread usage demands that the apparatus itself be simple in its construction and reliable in its operation.

#### CONCLUDING REMARKS

Apparatuses for the continuous culture of microorganisms may be thought of as devices for stabilizing a microbial population at a given growth phase. The system described above as internally controlled maintains the organisms indefinitely in the exponential phase and at the maximum growth rate characteristic of the nutrient medium and population size employed. In the externally controlled system the population also grows exponentially but at some chosen reduced rate.

With these techniques the changes in physiological state of a population which are the usual consequences of growth are avoided. And except for mechanical difficulties it might be expected that a population could be kept growing indefinitely. There is, of course, a possibility that a microbial culture cannot continue indefinitely to grow exponentially, but no convincing evidence of this has been presented.

However, it cannot be said that a culture growing in a continuous system is ageless. As time passes, mutations will occur. Those mutants that grow faster than the original type will therefore grow more rapidly than the washing-out rate, and will thereby increase in number in the population. In a continuous flow system operating according to the principle of internal control, the faster growing strain will eventually cause the washing-out rate to increase to a rate equal to the growth rate of the faster strain. This increase in washing-out rate will cause the original population to be completely washed-out of the growth tube. Similarly, in the externally controlled case a faster growing strain will displace the original population. This will occur as the faster strain brings about a reduction in the concentration of the controlling growth factor to a level where the growth rate of the faster strain equals the washing-out rate. Under these conditions the growth rate of the original strain will be less than the flow rate and that strain will be washed out of the growth tube.

Apparently a population inhabiting the growth tube cannot continue to live there indefinitely. As evolution in the form of mutation and selection is a necessary consequence of growth, continued growth of a population must eventually bring about its own destruction.

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## NUTRITION OF MICROORGANISMS<sup>1,2</sup>

By E. L. R. STOKSTAD, H. P. BROQUIST AND N. H. SLOANE

*American Cyanamid Company, Research Division, Lederle Laboratories,  
Pearl River, New York*

### INTRODUCTION

The study of bacterial nutrition continues to provide information of fundamental importance to the progress of biological chemistry. Several types of approach have been successfully used; one of these is the nutritional approach to metabolic problems, and another is the study of the nutritional requirement of various classes of organisms. The use of antimetabolites or metabolic antagonists has been a valuable approach in providing fundamental information on bacterial metabolism.

### GENERAL NUTRITIONAL REQUIREMENTS OF MICROORGANISMS

A detailed survey was reported by MacLeod & Onofrey (1) on the nutritional requirements of 34 marine bacteria isolated from sea water. Most of the organisms belonged to the genera *Mycoplana*, *Corynebacterium* and *Flavobacterium*. The majority grew in simplified medium containing only inorganic ions and carbon sources such as lactic, citric, or succinic acids. A few required amino acids, one required biotin, another biotin plus thiamine. One organism required an unidentified factor contained in yeast extract.

Russell, Bhandari & Walker (2) studied the vitamin requirements of 34 lactic acid bacteria isolated from brewery materials and found that pantothenic acid, nicotinic acid, riboflavin, and thiamine were the only vitamins required. Some required only one vitamin, such as pantothenic acid, while others required all four.

The requirements of the Reiter treponeme were studied by Steinman, Oyama & Schulze (3) who found that biotin, nicotinic acid, and pantothenic acid were essential. Thiamine and choline increased both the amount and the rate of growth while riboflavin was only stimulatory. These same investigators (4) found that the requirements of the saprophytic strain of *Treponema pallidum* were more complex and included serum albumin, carbon dioxide, cocarboxylase, leucovorin, coenzyme A, and pyruvic acid. Thiamine monophosphate was less than 0.1 per cent as active as cocarboxylase. Dihydro and tetrahydrofolic acids were 0.1 and 5 per cent as active as leucovorin while folic acid proved to be toxic to the organism.

<sup>1</sup> The survey of literature pertaining to this review was concluded in December, 1954.

<sup>2</sup> The following abbreviations will be used: ATP for adenosinetriphosphate; RNA for ribonucleic acid; DNA for deoxyribonucleic acid; HDP for hexose diphosphate; PABA for p-aminobenzoic acid; CF for citrovorum factor.



VITAMIN B<sub>12</sub>

The chemical relationship between the principal forms of vitamin B<sub>12</sub> and their activities for certain microorganisms is shown in Table I. The chemistry and biological relationships of vitamin B<sub>12</sub> have recently been reviewed by Hutner & Ford (5). Cyanocobalamin, vitamin B<sub>12</sub>, is the cyano-complex of vitamin B<sub>12</sub> which contains 5,6-dimethylbenzimidazole. A number of other forms have been reported which have been variously designated pseudo-vitamin B<sub>12</sub> (6) and Factors A, B, and C which were obtained from calf feces (7, 8). Pseudo-vitamin B<sub>12</sub> (9) contains adenine in place of the dimethylbenzimidazole. Factor B is the non-nucleotide portion of cobalamin. It has been isolated from hog feces (7) and derived chemically from cyanocobalamin (10) and from Factor A and pseudo-vitamin B<sub>12</sub> (11) by hydroly-

TABLE I  
PRINCIPAL FORMS OF VITAMIN B<sub>12</sub>

Compound	Nucleotide Base	Biological Activity		
		<i>L.</i> <i>leichmannii</i>	<i>E.</i> <i>coli</i>	<i>Ochromonas</i>
Vitamin B <sub>12</sub> (cyanocobalamin)	5,6-dimethylbenzimidazole	+	+	+
Pseudo-vitamin B <sub>12</sub>	Adenine	+	+	—
Factor A	2-methyladenine	+	+	—
Factor B	None	—	+	—
Factor G	Hypoxanthine	±	+	—
Factor H	2-methylhypoxanthine	±	+	—

sis with concentrated hydrochloric acid at 65° for 5 min. Factor B is three times as active as cyanocobalamin by pad plate assay with *E. coli* mutant 113-3, less active by the *E. coli* tube assay and inactive for *Lactobacillus leichmannii*.

Factor A (vitamin B<sub>12m</sub>), obtained from calf feces (12), is active for *E. coli* 113-3, *L. leichmannii*, *Euglena gracilis* but inactive for *Ochromonas malhamensis*. Factor A has been found by Brown & Smith (13) to contain 2-methyladenine in the nucleotide portion of the molecule. Deamination of Factor A by nitrous acid yielded a neutral crystalline substance from which 2-methylhypoxanthine could be obtained. This deaminated Factor A, which is active for *E. coli* 113-3 but only slightly active for *L. leichmannii*, appeared to be identical with a compound isolated from pig feces and tentatively designated Factor H. Deamination of "pseudo-vitamin B<sub>12</sub>" yielded a neutral crystalline product from which hypoxanthine could be obtained. This deaminated B<sub>12</sub> derivative was active for the mutant *E. coli*, and slightly

active for *L. leichmannii*; it appeared to be identical with Factor G which was isolated in traces from pig feces.

Vitamin B<sub>12f</sub> has been obtained from rat feces [Lewis *et al.* (14)]. Its exact chemical nature has not been established although it appears to be identical with pseudo-vitamin B<sub>12</sub> or with Factor A or be a mixture of the two [Ford *et al.* (15)]. It is active for *L. leichmannii* but inactive for the rat.

The vitamin B<sub>12</sub> requiring *E. coli* mutant is able to combine nucleotides and bases with Factor B to give various vitamin B<sub>12</sub> compounds. Ford & Holdsworth (16) thus obtained cyanocobalamin, Factor A, pseudo-vitamin B<sub>12</sub>, and cyanocobalamin from Factor B, by growing *E. coli* in a medium containing Factor B plus the corresponding nucleotides of these vitamins. Pseudo-vitamin B<sub>12</sub> was also obtained with adenine but not with adenosine, adenylic acid, or ATP. This indicates that the carbohydrate in the nucleotide from pseudo-vitamin B<sub>12</sub> is not the same as in adenylic acid and thus is not the  $\beta$ -form. Addition of riboflavin was also found to increase synthesis of cyanocobalamin from Factor B. More efficient synthesis was obtained with parts of the riboflavin molecule such as 1,2-dimethyl-4,5-diaminobenzene, or with the corresponding 5,6-dimethylbenzimidazole. These observations are in accord with the previous suggestion by Woolley (17) that 1,2-dimethyl-4,5-diaminobenzene is a common precursor of both riboflavin and cyanocobalamin.

The ability of *E. coli* to incorporate bases and nucleotides with Factor B has been utilized to produce new vitamin B<sub>12</sub>-like compounds containing compounds structurally related to benzimidazole or adenine. Ford, Holdsworth & Kon (18) obtained a "new vitamin B<sub>12</sub>" by culturing with benzimidazole. By using 5,6-dichlorobenzimidazole a new vitamin B<sub>12</sub> was formed which had approximately the same order of activity as cyanocobalamin for *E. coli*, *L. leichmannii* and *O. malhamensis*. New vitamin B<sub>12</sub> compounds were also formed with 5-methylbenzimidazole, 5-nitrobenzimidazole, 4-chloro-1,2-benzotriazole, benzthiazole, and 2,8-dichloroadenine. These were all active for *E. coli* and varying extents for *O. malhamensis*. The vitamin B<sub>12</sub> compounds formed with 5-aminobenzimidazole and 2,6-diaminopurine were active for *E. coli* but not for *O. malhamensis*. It thus appears that the biochemical defect in the *E. coli* mutant resides in its ability to synthesize the non-nucleotide moiety of vitamin B<sub>12</sub>. Similarly, Fantes & O'Callaghan (19) found that the addition of *o*-phenylenediamine during the fermentation of *Streptomyces griseus* resulted in the production of a new vitamin B<sub>12</sub>-like compound at the expense of cyanocobalamin. The biosynthesis of the new compound was prevented by the simultaneous addition to the medium of relatively low levels of 1,2-diamino-4,5-dimethylbenzene. The new vitamin B<sub>12</sub> compound is approximately twice as active as cyanocobalamin for *E. coli* and for *L. leichmannii*. This compound is in all probability the same as that obtained by incubation of *E. coli* mutant with Factor B and benzimidazole (18). This new compound, which has a pattern of microbiological activity for *E. coli*, *L. leichmannii*, and *O. malhamensis* similar to that of

cyanocobalamin, was reported by Fantes & O'Callaghan (19) to be active in three patients with pernicious anemia.

Vitamin B<sub>12</sub> has been found to increase the synthesis of DNA in organisms which do not require this vitamin for growth. Rege & Sreenivasan (20) studied the effect of folic acid and vitamin B<sub>12</sub> on the synthesis of DNA and RNA in *Lactobacillus casei* and *Streptococcus faecalis* which require only folic acid for growth, in *Lactobacillus leichmannii* which requires only B<sub>12</sub>, and in *E. coli* and *Lactobacillus arabinosus* which require neither. *L. casei*, grown on a medium partially deficient in folic acid, had less than half the DNA content of cells grown with both folic acid and vitamin B<sub>12</sub>. Addition of either vitamin increased the DNA to approximately 75 per cent of the normal level, although the cell mass and acid production were increased only by the addition of folic acid. The RNA content was not affected by a partial folic acid deficiency or by the addition of vitamin B<sub>12</sub>. Thymine had the same effect as folic acid on the DNA content of the cell. In a medium deficient in purines and pyrimidines both the RNA and DNA were reduced and both were increased by the addition of folic acid. Vitamin B<sub>12</sub> produced a further increase in DNA but had no effect on RNA. In *L. leichmannii*, which requires vitamin B<sub>12</sub>, a deficiency of this vitamin decreased growth and DNA content of the cells by approximately 40 per cent but was without effect on the RNA content. In the absence of purines a vitamin B<sub>12</sub> deficiency resulted in a reduction of both DNA and RNA, which were partially restored by addition of this vitamin. Addition of hydrolyzed DNA gave an increased cell mass and increased both DNA and RNA to their normal levels. With *L. leichmannii*, vitamin B<sub>12</sub> was able largely to reverse the action of aminopterin in lowering the DNA content of the cells. In *E. coli* and *L. arabinosus*, which do not require either folic acid or vitamin B<sub>12</sub>, these two vitamins were without effect on either DNA or RNA.

Other experiments by the same workers, Rege & Sreenivasan (21), showed that a level of chlortetracycline, which did not appreciably reduce cell mass, markedly reduced DNA. This effect was largely counteracted by increasing the level of folic acid or vitamin B<sub>12</sub>.

Two vitamin B<sub>12</sub> fragments, 1,2-dimethyl-4,5-diaminobenzene and 5,6-dimethylbenzimidazole, were found by Hallinger, Silber & Neumann (22) to inhibit the growth of *Mycobacterium tuberculosis* at levels of 16 and 33 µg./ml. respectively. The reversibility of this inhibition by vitamin B<sub>12</sub> was not studied. These two compounds were previously found by Hendlin & Soars (23) to inhibit growth of the vitamin B<sub>12</sub>-requiring organisms, *L. lactis*, *L. leichmannii*, and *Euglena gracilis*. The inhibition by 1,2-dimethyl-4,5-diaminobenzene was reversible by vitamin B<sub>12</sub> while the inhibition by dimethylbenzimidazole was non-reversible.

The observation by Hutner *et al.* (24) that *Ochromonas malhamensis* and two other chrysomonads require cobalamin prompted the suggestion that this might be a characteristic feature of this class of organisms. This view has received added support from the report by Droop (25) that three other

chrysomonads, *Monocrysis lutheri* Droop, *Prymnesium parvum* Carter and *Syracosphaera elongata*, have an absolute requirement for vitamin B<sub>12</sub> and a partial requirement for thiamine. Half maximum growth is given by approximately 0.01  $\mu$ g/m. of vitamin B<sub>12</sub> per ml. and is not influenced by the presence of either methionine or nucleotides, or their corresponding bases.

The ability of the root nodule bacteria, *Rhizobium meliloti*, *R. trifolii*, and *R. leguminosarum* to synthesize vitamin B<sub>12</sub> *in vitro* and *in vivo* within the root nodule has been confirmed by Levin *et al.* (26).

The role of vitamin B<sub>12</sub> in the reduction of homocystine has been studied by Dubnoff (27), using an *E. coli* mutant requiring vitamin B<sub>12</sub>. This organism previously had been shown to respond to either vitamin B<sub>12</sub>, methionine, homocystine, or compounds capable of reducing homocystine to homocystine. On the basis of this it was postulated that one of the functions of vitamin B<sub>12</sub> is the reduction of homocystine to homocystine (28). In the recent investigation (27), it was found that a series of mutants with varying B<sub>12</sub> requirements could be isolated from the original *E. coli* mutant 113-3. The variant with the smallest B<sub>12</sub> requirement grew maximally on as little as 2  $\mu$ g. of homocystine or its thiolactone while 10  $\mu$ g. of methionine was required to produce the same response. This suggests that homocystine acts in catalytic amounts and not solely as the carbon skeleton of methionine. The strain with the highest B<sub>12</sub> requirement did not respond to homocystine or its thiolactone. The lowest B<sub>12</sub>-requiring mutant responded submaximally to PABA while PABA had no effect on the highest B<sub>12</sub>-requiring strain. A third strain with intermediate B<sub>12</sub> requirement gave a small response to homocystine but the B<sub>12</sub> requirements were greatly reduced by the addition of this amino acid. The trace requirement for B<sub>12</sub> in the presence of homocystine could be replaced by a combination of PABA and dimethyl- $\beta$ -propiothetin. A fourth mutant could not be subcultured continuously on methionine, showing that B<sub>12</sub> is required for reactions other than methionine synthesis in this organism.

#### FOLIC ACID-CITROVORUM FACTOR

Considerable attention is currently being given to the exact chemical nature of the folic acid coenzyme involved in the metabolism of the one-carbon unit. Most of the recent advances in this field have come from detailed studies of purified enzyme systems from pigeon liver rather than from studies of bacterial nutrition. Hence this subject will not be dealt with in detail here.

The studies of Greenberg (29) on the nature of the folic acid coenzyme catalyzing the incorporation of formate into 5-amino-4-imidazolecarboxamide-5'-phosphoribotide in a pigeon liver enzyme system demonstrated that 5-formyl-5,6,7,8-tetrahydrofolic acid (leucovorin) per se was not the active cofactor, but leucovorin was "activated" following preincubation with ATP and a liver fraction. Studies by Kisluk & Sakami (30, 31) as well as by Blakely (32) on the glycine-serine interconversion in pigeon liver enzyme

systems demonstrated that tetrahydrofolic acid was the most active folic acid derivative tested. These workers speculated that  $N^5$ -hydroxymethyl-tetrahydrofolic acid (30, 31) or the tetrahydroglyoxaline derivative (32) in which formaldehyde condenses with both the  $N^5$  and  $N^{10}$  positions of tetrahydrofolic acid might be the active cofactor in the serine-glycine system. If the active coenzyme forms of folic acid are labile, it may be difficult to obtain evidence for their existence from nutritional studies with microorganisms. Lascelles & Woods (33) have studied the synthesis of serine by resting cells of *Streptococcus faecalis* R. Serine synthesis was dependent on the presence of glycine, formate, glucose, pyridoxal, and folic acid. It was observed that during the synthesis of serine, a factor was formed which was active for *Leuconostoc citrovorum* but which did not appear to be identical with leucovorin. Additional evidence for the involvement of folic acid in serine synthesis in microorganisms has been found with *L. mesenteroides* P-60 by Lascelles and co-workers (34). This organism normally requires serine and PABA for growth. Serine could be omitted, however, if adequate glycine were added together with pyridoxal, PABA, and carbon dioxide. Leucovorin was found to replace both PABA and carbon dioxide in the serine-deficient medium. In the presence of serine the requirement of *L. mesenteroides* for PABA could be met by leucovorin. In contrast, neither folic acid nor  $N^{10}$ -formylfolic acid could replace PABA under conditions in which leucovorin was active. From these observations it seemed likely that *L. mesenteroides* converted either PABA or leucovorin to the active folic acid coenzyme, but folic acid did not appear to be a direct intermediate on this pathway.

Koft & Morrison reported briefly (35) that *Acetobacter suboxydans*, which requires PABA, produces a metabolite of unknown nature which replaces the folic acid requirement of *Streptococcus faecalis* and *Lactobacillus casei*, and the PABA requirement of *L. plantarum*. Virtanen & Lundbom (36) reported that *Clostridium pasteurianum*, a nitrogen fixing anaerobe, requires folic acid.

Bolinder and co-workers (37) studied the conditions for the enzymatic digestion of yeast extract and synthetic conjugates of leucovorin by the folic acid conjugases in chicken pancreas and hog kidney. The end products of the digestion of these substrates were predominately the diglutamate of 5-formyltetrahydroptericoic acid when chicken pancreas was employed and the monoglutamate with hog kidney. When yeast extract was digested with chicken pancreas (pH 5.5) or with hog kidney (pH 4.5), a growth factor, termed "Factor I," active for growth of *S. faecalis*, *L. casei*, and *Leuconostoc citrovorum*, was formed. "Factor I" could be formed chemically by treating leucovorin with alkali, or by boiling leucovorin with a number of reducing agents or serine, but was apparently not identical with known folic acid derivatives. Ericson (38) used the bioautographic methods of Bolinder *et al.* (37) to examine the folic acid content of 17 marine algae. Bioautographic evidence indicated that formylated or formylated and reduced derivatives

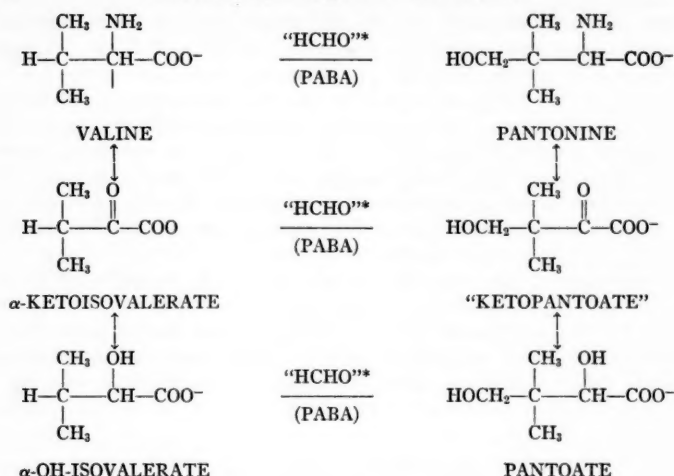
of folic acid predominated; the di- and triglutamate of 5-formyltetrahydroptericoic acid were recognized. The report of Felton & Niven (39) that *Leuconostoc citrovorum* strain 8081 is a member of the genus *Pediococcus* has been confirmed by Jensen & Seeley (40). The latter workers studied 34 strains of the genus *Pediococcus*; all of these strains had an absolute requirement for 5-formyltetrahydrofolic acid. Nichol (41) has studied the enzymatic formation of citrovorum factor (CF) from folic acid by cell free extracts of an A-Methopterin-resistant strain of *Streptococcus faecalis*. In addition to glucose, ascorbate, and formate or serine which markedly stimulate CF formation from folic acid by intact cells (42), a requirement for  $Mg^{++}$ , DPN, and ATP was demonstrated. The conversion of folic acid to CF was inhibited by minute amounts of Aminopterin or A-Methopterin in the cell free enzyme system in contrast to large amounts of these antagonists needed to block this reaction when carried out with intact cells. It was suggested that a major factor in the acquisition of resistance to this class of folic acid antagonists may be in a reduction in the permeability of the bacterial cell to the antagonist.

#### PARA-AMINOBENZOIC ACID

Purko, Nelson & Wood (43) described the role of PABA in pantoate synthesis by *Bacterium linens*. The ability of PABA to substitute for pantothenic acid in the growth of this organism indicated that PABA may function in the synthesis of this factor. Both folic acid and leucovorin were ineffective in replacing PABA which was necessary for growth in the absence of pantothenic acid. Pantoic acid and ketopantoic acid were found to be as active as PABA in promoting growth of the organism; and  $\alpha$ -ketoisovaleric acid exerted a PABA-sparing effect. Pantoinine,  $\alpha$ -ketoisovaleric acid,  $\alpha$ -hydroxyisovaleric acid, and valine were also implicated in pantoic acid synthesis, since these compounds were weak competitive reversing agents of salicylate inhibition in the presence of PABA; in this system pantoate and ketopantoate were active, non-competitive reversing agents.

The results of this investigation are in accord with the pathway of biosynthesis postulated by Kuhn & Wieland (44) as involving  $\alpha$ -ketoisovalerate, ketopantoate, and pantoate. The results are also in agreement with those of Lansford & Shive (45) and Maas & Vogel (46), concerning the roles of ketopantoate and  $\alpha$ -ketoisovalerate, respectively, as intermediates in pantothenate synthesis. The interrelations suggest that PABA functions in the hydroxymethylation of  $\alpha$ -ketoisovalerate to ketopantoate. The involvement of PABA in pantoate synthesis is shown in Fig. 1.

Sloane (47, 48) reported the isolation of a crystalline metabolite of PABA which (non-competitively) reversed the inhibitory effect of chlortetracycline and oxytetracycline on the hydroxylation of aniline by resting cell of *Mycobacterium smegmatis* or *M. tuberculosis* 607. The metabolite appears to function as a cofactor or cosubstrate in the hydroxylation reaction. The compound does not affect the antibiotic activity of the tetracyclines and the degrada-



\* Hydroxymethylation reaction.

FIG. 1. The Role of Paba in Pantothenate Biosynthesis.

tion products of chlortetracycline which are biologically inert do not effect the hydroxylation reaction. The role of the metabolite in relation to the function of PABA is yet to be determined.

#### PANTHOTHENIC ACID

Pierpoint & Hughes (49) determined that washed cells of *L. arabinosus* take up pantothenate when suspended in glucose-buffer medium containing  $\text{Mg}^{++}$ . The cells were found to synthesize 50 to 100 units of coenzyme A per gram of dry weight. The addition of cysteine or cystine increased the synthesis of coenzyme A by 6 to 8 fold. Pantetheine completely replaced both pantothenate and cystine in the synthesis of Co A, which indicated that the role of the amino acid was in the formation of the  $\beta$ -mercaptoethanol residue of Co A, as previously indicated by Brown & Snell (50). In the absence of cysteine, washed suspensions of pantothenate-deficient cells concentrated pantothenic acid from the suspending medium; it was released again upon treatment with alkaline phosphatase. These data suggest that pantothenate is phosphorylated in the absence of cysteine.

Hall *et al.* (51) determined that 11 pantothenate-requiring strains of acetic acid bacteria could utilize pantolactone in lieu of pantothenic acid.

Lindan (52) determined that the inhibitory effect of  $\beta$ -aminobutyric acid on *Saccharomyces carlsbergensis* 4228 is reversed by pantothenate. It was concluded that the inhibition could be due to the formation of  $\alpha$ -methylpantothenate which would interfere with pantothenate utilization.



Altenbern & Ginoza (53) studied pantothenate synthesis by a smooth strain of *Brucella abortus*. The organism coupled pantoyllactone and  $\beta$ -alanine to produce pantothenate, after an initial lag phase. The addition of valine or  $\alpha$ -ketoisovalerate in place of the lactone resulted in immediate pantothenate synthesis, but the rate of the reaction was considerably diminished. The addition of L-serine to starved cells increased the rate of pantothenic acid synthesis from L-valine or ketovaline; D-serine inhibited the reaction. The inhibition of the biosynthesis caused by the addition of L-leucine and L-isoleucine was thought to be due to the interference at the transformylation step which converts ketovaline to ketopantoate, since these amino acids did not inhibit the coupling of pantoyllactone and  $\beta$ -alanine. Under proper conditions either L-asparagine or L-aspartic acid increased pantothenic acid synthesis by furnishing additional  $\beta$ -alanine. The ability of valine or  $\alpha$ -ketoisovalerate to act as precursors in pantothenate biosynthesis is in accord with the observations of Purko *et al.* (43).

Brown & Snell (54) studied the effect of pantothenic acid conjugates on the growth of pantothenic acid-requiring *Acetobacter suboxydans*. The activities of 4'-phosphopantethine, coenzyme A, pantethine, and pantothenylcysteine were increased markedly by autoclaving with the medium; the —SH form of the compound was determined to be the active growth factor. Autoclaving effected partial reduction of the added compound. Coenzyme A and the pantethine degradation products had greater activity for the organism than pantothenic acid itself.

Dewey & Kidder (55) found that pantothenic acid was more active in promoting growth of *Tetrahymena* and *Colpidium* than were any of the conjugated forms of the vitamin; but pantethine was the most active form of the compound for promoting growth of *Glaucoma*.

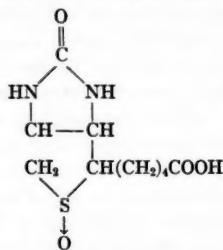
Drell & Dunn (56, 57) determined that  $\omega$ -substituted analogues of pantothenic acid inhibited the growth of lactic acid bacteria. The  $\omega$ -methyl analogue was the most inhibitory compound of the series.

#### BIOTIN

Wright and co-workers in a series of papers (58, 59, 60) have reported the occurrence, isolation, and characterization of a previously unrecognized form of biotin. When *Aspergillus niger* was grown on a medium supplemented with pimelic acid, a growth factor termed AN factor, having biotin-like activity for *Neurospora crassa*, was produced (58). A few milligrams of AN factor was isolated from *A. niger* fermentation liquor (59) and shown to be biotin-1-sulfoxide (60).

The possibility that biotin-1-sulfoxide is an artifact arising by oxidation of biotin during the aerobic fermentation by *A. niger* was ruled out (60), which implies that the sulfoxide is of metabolic significance. The two sulfoxides of biotin, the *dextro* and *levo* forms, were prepared chemically from biotin by Melville (61) and their biological properties studied (62). The *dextro* form was as active as biotin for *Lactobacillus arabinosus* and *Saccharomyces*

*cerevisiae*; in contrast the *levo* form was only 5 per cent and 0.1 per cent as active respectively for these organisms. Neither of the sulfoxides was effective in curing the biotin-deficiency syndrome for rats when fed at 100 times the effective level of biotin. Wright & Driscoll (63) demonstrated by paper chromatographic procedures that *Aspergillus niger* utilizes desthiobiotin to form biotin-L-sulfoxide, implying that the biosynthetic pathway



Biotin-1-sulfoxide

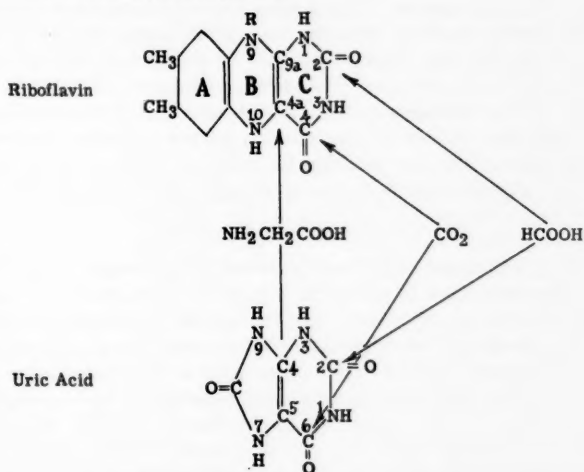
from desthiobiotin proceeds through biotin. During the course of the investigations leading to the discovery of biocytin ( $\epsilon$ -N-biotinyl-L-lysine) and biotin-L-sulfoxide, many bioautographic data on the different known forms of biotin were obtained by Wright and co-workers. These data have been summarized (64) for biotin, desthiobiotin, oxybiotin, biocytin, biotin-L-sulfoxide, and biotin-D-sulfoxide. Wright and co-workers (65) have reported the existence and properties of an enzyme in blood, termed biocytinase, which cleaves biocytin hydrolytically to yield biotin.

In contrast to an earlier report (66), Wessman *et al.* (67) reported that biotin is essential for growth of *Micrococcus lysodeikticus*. The biotin could be replaced by sufficient oleic acid plus aspartic acid. No evidence for the involvement of biotin as a cofactor for oxalacetic acid decarboxylase was found, but biotin was involved in the fixation of carbon dioxide into oxalacetate by this organism. Schaefer *et al.* (68) briefly reported that a strain of *Mycobacterium tuberculosis* which requires biotin and can normally be grown in air, is unable to grow when biotin is replaced by oleic acid unless it is cultured in an atmosphere of carbon dioxide. A similar relationship between biotin and carbon dioxide was earlier reported for *Lactobacillus arabinosus* by Broquist & Snell (69). Hofmann and co-workers have reported (70) on the biotin-like activity for several lactobacilli of lactobacillic acid and a number of other synthetic and naturally occurring long chain fatty acids possessing a cyclopropane ring (71). An excellent review has appeared summarizing present knowledge concerning fatty acids and growth of microorganisms (72).

#### RIBOFLAVIN

Several communications have appeared on the mechanism of microbial synthesis of riboflavin. The earlier finding of MacLaren (73) that a

number of purines were effective in increasing riboflavin synthesis by *Eremothecium ashbyii* has been confirmed and extended by several workers. McNutt (74) reported that several purine nucleosides and nucleotides were no more active than the free bases in stimulating riboflavin formation by this organism. When adenine, randomly labeled with  $C^{14}$ , was added to a growing culture of *E. ashbyii*, the radioactivity was found principally in the 6,7-dimethylisalloxazine portion of the riboflavin. Carbon atom 8 of adenine was not significantly incorporated into riboflavin. In a similar study, Goodwin & Pendlington (75) observed that threonine, serine, and tyrosine, as well as the purines, stimulated riboflavin biosynthesis by *E. ashbyii*, the effects of the purines and the amino acids being cumulative. These workers proposed that threonine or serine might function as the "aromatic precursor" of riboflavin and that the purine ring might be incorporated intact. Klung-søyr (76) studied riboflavin formation by resting cells of *E. ashbyii*. A small amount of  $C^{14}$  carboxyl-labeled acetic acid was incorporated into the vitamin. By degradation procedures it was shown that the  $C^{14}$  activity resided in carbon atom 2 of riboflavin. The interpretation of these findings has become clearer as a result of the experiments of Plaut *et al.* (77, 78), who have studied the incorporation of several simple radioactive compounds into riboflavin by *Ashbyii gossypii*. The accompanying diagram (77) summarizes the salient points of Plaut's work and shows biosynthetic pathways common to the synthesis of riboflavin and purines.



$C^{14}$  formate was incorporated to the extent of 95 per cent into the 2-carbon position.  $C^{14}$  bicarbonate was found predominantly in the 4-carbon position. When either  $NH_2C^{14}H_2COOH$  or  $NH_2CH_2C^{14}OOH$  was added to growing cultures, nearly all of the radioactivity in the vitamin resided in

carbon atoms 4a and 9a. Elegant degradation procedures developed by Plaut were essential for the foregoing work; subsequently, degradation procedures for the o-xylene portion of ring A of the vitamin have appeared, thus permitting a study of the origin of these C-atoms from glucose (78).

Burgess *et al.* (79) reported that a pteridine isolated from developing grasshopper's eggs replaced riboflavin for a number of lactobacilli. Further details are awaited with interest. A chemical synthesis of D-riboflavin-2-C<sup>14</sup> starting with urea-C<sup>14</sup> was reported (80); the product was utilized by growing *Lactobacillus casei* cells for the formation of flavin adenine nucleotides and an unidentified compound.

#### NICOTINIC ACID

Yanofsky (81) investigated the pathway of niacin synthesis in bacteria using nicotinic acid auxotrophs of *E. coli* and *Bacillus subtilis*. The known intermediates from tryptophan which were effective for niacin synthesis by *Neurospora* were without effect in supporting the growth of these mutants. The compounds tested were: anthranilic acid, indole, tryptophan, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, quinolinic acid, kynurenic acid, xanthurenic acid, and ornithine. Furthermore, tryptophan auxotrophs were unable to convert kynurenine to anthranilic acid (which supported growth of the mutants); this finding suggested that the strains lacked kynureninase (which converts kynurenine to anthranilic acid) and is consistent with the view that these strains are unable to convert tryptophan to niacin. Further evidence that the pathway of niacin synthesis does not involve tryptophan was obtained by growing the *E. coli* auxotroph in the presence of C<sup>14</sup>-tryptophan or C<sup>14</sup>-indole and isolating the nicotinic acid formed; it did not contain any C<sup>14</sup>. The C<sup>14</sup>-indole was, however, converted to C<sup>14</sup>-tryptophan without isotope dilution by a tryptophan auxotroph of *E. coli*. Therefore, it is apparent that the pathway of niacin biosynthesis in these bacteria differs from the pathway employed by *Neurospora* (82).

#### THIOCTIC ACID (LIPIC ACID)

Barker (83) reported that *Butyribacterium rettgerii* requires thioctic acid as a growth factor when lactate is the energy source, but not when glucose or pyruvate is the energy source. The requirement was approximately 0.1  $\mu$ g. of DL-thioctic per ml. of medium for half maximum growth. Since pyruvate is converted to acetic acid, butyric acid, and carbon dioxide in the absence of thioctic acid, thioctic acid is not essential for the decomposition of pyruvic acid. Growth with glucose in the absence of added thioctic acid is not due to a synthesis of thioctic acid, since such cells do not contain this factor. Furthermore, when this organism is grown in the presence of both glucose and lactate, there is no decomposition of the lactic acid. The authors conclude that thioctic acid is not needed for the decomposition of pyruvate but is required either for the utilization of lactate or for the penetration of lactate into the cell. Further evidence against the participation of thioctic

acid in the utilization of pyruvate was provided by the observation that arsenite, which combines with thioctic acid, had no effect on anaerobic pyruvate decomposition by resting cell suspensions. Arsenite did inhibit lactate decomposition at a concentration of  $10^{-6}$ M.

Analogues and homologues of thioctic acid have been prepared and examined for growth promoting and antagonist activities [Stokstad (84)]; 8-methylthioctic acid (DL-6,8-dithiooctanoic acid) behaved as a reversible antagonist and had an inhibition index of 30,000 to 1 for the growth of *S. faecalis*, 100 to 1 for *Tetrahymena geleii*, and 100 to 1 for the pyruvate oxidation by resting cells of *S. faecalis*. This compound had slight growth promoting activities for *Corynebacterium bovis*. It was inactive against *E. coli*. Shifting the secondary sulfur in thioctic acid to the 4,5 or 7 position decreased the activity to between 0.1 and 1 per cent of that of thioctic acid. An analogue containing only one sulfur atom (8-benzylthio-6-ketooctanoic acid) was 1 per cent as active as thioctic acid for *T. geleii*.

Baker, Sobotka & Hutner (85) determined the growth requirements of mesophilic and thermophilic strains of *Bacillus licheniformis* and *B. circulans*. There were differences in the energy and amino acid and vitamin requirements of the mesophilic and thermophilic strains. The mesophilic strain of *B. licheniformis* did not respond to thioctic acid under any conditions, whereas the thermophilic strain on glycerol-containing media responded to a combination of acetate plus thioctic acid (0.4  $\mu$ g./ml.). Growth of the thermophilic strain could also be obtained in the absence of thioctic acid by the addition of certain citric acid cycle intermediates, such as malate (up to 0.1 per cent), or a combination of citrate (0.05 per cent) with either acetate (0.01 per cent) or fumarate (0.1 per cent). Acetate or fumarate alone did not support growth. Sodium bicarbonate, added aseptically to the glycerol medium, permitted dense growth in the absence of thioctic acid or any citric acid intermediates. Thiamine (1  $\mu$ g./ml.) could replace the thioctic acid requirements (0.4  $\mu$ g./ml.) with this thermophilic organism.

The effectiveness of bicarbonate in replacing the thioctic acid requirement of this thermophile is similar to that observed by Lytle, Zulick & O'Kane (86) in *S. faecalis* 734. This organism grew submaximally with glucose but was stimulated by acetate or thioctic acid. With gluconic acid as the energy source, no growth occurred in the absence of thioctic acid or acetate. The acetate could be replaced by either malate, succinate, or bicarbonate.

#### THIAMINE

The thiamine requirement of *Tetrahymena geleii* was found by Tittler & Bovell (87) to be partly met by 100  $\mu$ g./ml. of sulfadiazine, the maximal growth obtainable with sulfadiazine being half that with thiamine. Sulfadiazine also prevented the accumulation of pyruvic acid which occurred on the thiamine-deficient media. Other sulfonamides such as sulfamerazine, sulfathiazole, sulfanilamide, and sulfapyridine were ineffective.

## PURINES AND PYRIMIDINES

The research emphasis in this area has been placed on the biosynthetic pathways of pyrimidine and purine base synthesis as carried out by growing and resting cultures. Dihydroorotic acid and ureidosuccinic acid support the growth of several pyrimidineless lactobacilli (88) but dihydrouracil and ureidopropionic acid were found to be inactive (89), which further emphasizes the specificity of orotic acid for pyrimidine synthesis. The orotic acid requirement of *L. bulgaricus* 09 could be met by approximately equimolar amounts of uridine-5'-phosphate, polynucleotides containing uridine-5'-phosphate as a component, and uridine-2'3'-phosphate (90). Rege & Sreenivasan demonstrated that resting cells of *Bacillus subtilis* convert uracil to thymine (91). Compounds that might function as donors of the 1-carbon unit for the 5-methyl group of thymine were tested. Glycine and to a lesser extent serine, threonine, and sarcosine increased the formation of thymine. This transformation in *B. subtilis* requires further investigation; more detailed study might show the involvement of orotic acid derivatives in this reaction. Weed & Wilson (92) have shown that when ureido carbon labeled ureidosuccinic acid, methylene-labeled aspartic acid, and methylene-labeled oxalacetic acid are added to growing cultures of wild type *E. coli*, these compounds are incorporated into uracil and cytosine, but not into adenine.

A number of amino acids have been implicated in pyrimidine biosynthesis through nutritional studies. Woods *et al.* (93) cultivated *Lactobacillus arabinosus* in a medium deficient in uracil and aspartic acid; growth was stimulated by the addition of either aspartate or uracil. Uracil spared the aspartic acid requirement and also overcame in a noncompetitive fashion the growth inhibitory action of cysteic acid, an aspartic acid antagonist. Orotic acid and ureidosuccinic acid were not active. It was inferred from these findings that aspartic acid is involved in the biosynthesis of uracil. Wahba & Shive (94), employing similar microbiological techniques, also implicated aspartic acid in the biosynthesis of purine bases by *L. arabinosus*. Fairley (95) found that threonine and  $\alpha$ -amino-n-butyric acid could support growth of various pyrimidineless mutants of *Neurospora crassa*, which suggested that these amino acids are concerned in pyrimidine synthesis by this organism (95).

Heinrich, Dewey & Kidder have presented conclusive evidence on the biogenesis of carbon atom #2 of pyrimidines in *Neurospora* (96). A strain of *Neurospora* (46004a) was grown in a pyrimidine-deficient medium with carbamyl labeled  $C^{14}$ -L-citrulline, and the purine and pyrimidine bases were isolated from the cell nucleic acids. Negligible radioactivity was present in adenine and guanine, but cytosine and uracil were rich in  $C^{14}$ . Degradation of uracil demonstrated the incorporation of the carbamyl carbon of citrulline into carbon 2. This mechanism may have been operative in the nutritional experiments of Newton & Wilson (97) who demonstrated that two strains of *Brucella abortus* fix carbon dioxide only into nucleic acid pyrimidines under their experimental conditions.

Holmes & Welch (98) have described a simple and useful pad plate microbiological assay procedure for thymine or thymidine. This procedure employs *Streptococcus faecalis* with a folic acid-deficient medium; sufficient Aminopterin is added to block interfering growth responses to folic acid derivatives.

Brooke *et al.* (99) studied the factors affecting the excretion of orotic acid by certain pyrimidineless and purineless mutants of *A. aerogenes*. The addition of uracil to the medium depressed the formation of orotic acid by the mutant, apparently by suppressing the *de novo* synthesis of orotic acid.

A number of analogues of orotic acid have been prepared and tested with several bacteria for growth promotion or inhibition. Prusoff (100) found that 5-iodoorotic acid and iodouridine would not replace folic acid, thymine, or thymidine for *Streptococcus faecalis* or *Lactobacillus casei*, nor were they antagonistic in the presence of these compounds (100). Although iodoorotic acid appeared to support growth of *L. bulgaricus* 09, it seemed more likely that growth was attributable to the decomposition of the iodo-compound to yield orotic acid. Holmes & Welch found 6-uracilsulfonamid and 6-uracilmethylsulfone to be inhibitory for *L. bulgaricus* 09 when grown with either orotic acid, ureidosuccinic acid, or 1-dihydroorotic acid (101). Preliminary data indicated that these antagonists inhibit the decarboxylation of orotic acid and hence its conversion to uridine-5-phosphate. These compounds should prove useful in biochemical studies to elucidate the function of orotic acid in various species.

Zamenhof & Griboff (102) isolated the DNA from certain strains of *E. coli* grown on 5-bromouracil and found that 18 to 28 per cent of the thymine of the DNA was replaced by the unnatural analogue. Prusoff (103) has studied the inhibitory action of 5-bromouracil on *S. faecalis*; its toxicity could be reversed competitively by thymine, or incompletely and non-competitively by thymidine. Evidence for the formation of a nucleoside of the analogue was presented.

Interest continues in the role of 4-amino-5-imidazolecarboxamide in the purine nutrition of microorganisms. A detailed study of the energy and nitrogen requirements for the biosynthesis of carboxamide by resting cell suspensions of a purine-requiring *E. coli* mutant has been made (104). Potassium ion has been reported (105) as stimulating the accumulation of the carboxamide in *E. coli*. A report by Aaronson & Nathan (106) indicates that 4-amino-5-imidazolecarboxamide may be a more immediate precursor of adenine than 4-amino-5-imidazolecarboxamide. The latter compound would yield hypoxanthine on introduction of formate while the corresponding carboxamide would give adenine. This conclusion is based primarily on the observation that the purine requirement of the protozoa *Crithidia fasciculata* could not be replaced by the carboxamide but one-half to three-fourths maximal growth was obtained with the carboxamide. Chamberlain & Rainbow (107) have reported that a diazotizable amine and hypoxanthine accumulate when *Saccharomyces cerevisiae* is grown in a



medium containing adequate methionine but suboptimal amounts of biotin. The amine is not 4-amino-5-imidazolecarboxamide or the amidine, but is capable of replacing adenine for a purine-requiring yeast and guanine for *Lactobacillus brevis*. Amine accumulation is depressed by adenine or by a number of amino acids, including aspartic acid. A similar purine precursor has been described by Love & Gots (108). They found that a purineless *E. coli* mutant (W-11) accumulates an imidazole-pentose derivative which can be converted to 4-amino-5-imidazolecarboxamide by cell suspensions of another *E. coli* mutant (B-96) which normally accumulates the carboxamide. Preliminary chemical evidence suggested that the compound accumulated by *E. coli* (W-11) is an aminoimidazole ribotide or riboside which functions as a precursor of the 4-amino-5-imidazolecarboxamide derivative.

Magasanik & Brooke (109) found that a guanineless mutant of *A. aerogenes* (P14) accumulates xanthosine when grown on guanine. Since inosine-5-phosphate appears to be the precursor of both the adenine and guanine of the bacterial nucleic acids, this accumulation suggests that inosinic acid is converted to nucleic acid guanine via a derivative of xanthine.

Elion and co-workers (110) have studied the synergistic action of nucleic acid and folic acid antagonists in *S. faecalis* and *L. casei*. A marked synergistic effect was noted in *S. faecalis* between folic acid antagonists and thymine antagonists (5-bromouracil, 6-azathymine). A graphical method of presenting the data was developed which permitted the degree of potentiation observed to be expressed numerically; this should be useful to workers in the field of chemotherapy. Nathan & Cowperthwaite (111) compared the toxic action of several 2,4-diaminopyrimidines for the protozoan *Crithidia fasciculata*, for a number of lactic acid bacteria and for two plasmodia; they concluded that *C. fasciculata* would be a useful organism for screening antimalarials.

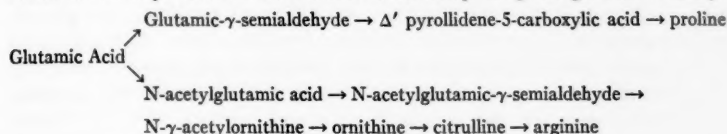
Barker & Kornberg (112) have reinvestigated the chemical nature of the adenosine triphosphate of *Thiobacillus thiooxidans*. They believe it to be the 5'-triphosphate, not the 2'- or 3'-triphosphate as reported earlier (113).

#### AMINO ACIDS

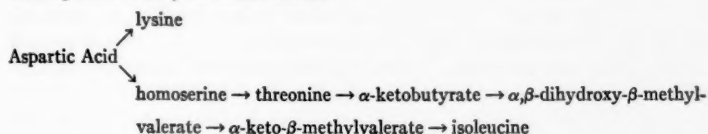
During the past year investigations on the effects of amino acids in the nutrition of microorganisms were mainly concerned with the interrelationships of amino acids and other essential factors. As in previous years the techniques employed in these studies were: (a) the use of mutant strains which require a single amino acid, (b) the use of the isotope techniques to trace the pathway of amino acid biosynthesis and to establish interrelationships of amino acids with other factors, and (c) reversal of growth inhibition produced by specific antagonists.

Abelson (114), in an extension of a preliminary report (115) on ornithine and proline synthesis, studied the pathways of amino acid biosynthesis in

*E. coli* by means of the isotopic dilution technique using randomly labeled  $C^{14}$ -glucose. Results obtained by using this technique verify the previous interrelationships between the amino acids comprising the glutamic family.



Vogel & Bonner (116) have also described the interrelationships between glutamate, proline and ornithine in *Neurospora*. Competition studies using  $C^{14}$ -glucose and unlabeled aspartic acid, demonstrated the interrelations of the aspartic family of amino acids.



Unlabeled pyruvate was found to have some effect on the incorporation of  $C^{14}$  from glucose into the glutamic and aspartic acid families of amino acids; the greatest effect was shown on alanine, valine, and leucine. The interrelations between serine and glycine, serine and cystine, and serine and pyruvic acid, were also shown by this method of analysis.

McQuillen & Roberts (117) studied the utilization of labeled acetate for synthesis in *E. coli*. In glucose-salts medium, *E. coli* was found to fix both acetate carbons into glutamic acid, proline, arginine, aspartic acid, threonine, lysine, methionine, isoleucine, and leucine. The specific activities of glutamic acid, proline, and arginine are so nearly equal as to suggest that these amino acids derive the same number of carbon atoms from acetate. The same relationship also appears between aspartic acid and threonine. The addition of unlabeled aspartate reduced incorporation of acetate carbon into aspartic acid, threonine, lysine, and methionine by 75 to 80 per cent. Glutamic acid abolished the acetate uptake into virtually all the amino acids except leucine. The addition of unlabeled proline, arginine, lysine, leucine, and methionine caused suppression of acetate incorporation into each individual amino acid. The results of this study suggest that two separate acetate fixation mechanisms are involved in amino acid biosynthesis: (a) one in which leucine is the product, and (b) one in which glutamic acid or a substance in equilibrium with it (e.g. ketoglutarate) is an early product. The high fixation of labeled acetate in the protein fraction of *E. coli* cells indicates that the Krebs cycle in actively growing cells serves mainly as part of a mechanism for synthesis of a group of amino acids. The interrelationships of many of these amino acids are clarified by the individual investigations reported in this section of the review.

*Neutral amino acids.*—The metabolism of phenylalanine and tyrosine by *E. coli* strain K-12 and two other mutants was studied by Simmonds and co-workers (118). Threo-phenyl-DL-serine inhibited the growth of these microorganisms and this inhibition was reversed more effectively by phenylalanine than by tyrosine, but the presence of tyrosine in the medium spared the phenylalanine requirement. During the secondary growth phase the phenylalanine auxotrophs were found to accumulate material with growth-promoting activity for the mutant. The factors in the filtrate responsible for the growth were isolated by means of paper chromatography; one factor was identified as phenylalanine, the second appeared to be a phenylalanine-containing peptide.

Fox & Warner (119) demonstrated that the growth antagonism between phenylserine and phenylalanine varied with the phenylserine isomer employed and the microorganisms studied. This investigation was concerned with the growth inhibitory effect of the analogues on *L. arabinosus* and *L. brevis*. With *L. arabinosus*, phenyl-DL-serine showed inhibition whereas the allo-phenyl-DL-serine exhibited virtually no inhibition. The D-isomer was shown to be the stronger antagonist. With *L. brevis*, phenyl-DL-serine and the allo-phenyl-DL-serine were shown to be phenylalanine antagonists.

Alanine has been found by Mika *et al.* (120) to cause population changes in strains of *Brucella* from the smooth to the rough type. The action of  $\alpha$ -alanine was correlated with an interference either with pantothenate synthesis or with its utilization. The effect of alanine was reversed by pantothenate. Salicylate, which has been shown to interfere with pantothenate synthesis (121), and pantothenic acid analogues such as  $\beta$ -aminopropiophenone, and DL-[(N-pantoyl) N-tauryl]p-anisidide also caused these population changes.

Adelberg (122) studied the biosynthesis of isoleucine from threonine by *Neurospora*, using acetate-1,2-C<sup>14</sup> and unlabeled threonine. Threonine suppressed the incorporation of radioactivity into carbons 1,2,4 and 5 of isoleucine. Furthermore, threonine-1,2-C<sup>14</sup> was found to be converted to the  $\alpha,\beta$ -dihydroxy-1,2-C<sup>14</sup> analogue of isoleucine. Strassman *et al.* (123) have proposed similar mechanisms for isoleucine biosynthesis by *Torulopsis utilis*. The distribution of C<sup>14</sup> in the carbon atoms of isoleucine formed from labeled acetate agreed remarkably well with the calculated distribution patterns based on the biosynthetic pathway postulated by Adelberg (122).

The nutritional requirements for reproduction of *E. coli* phage T2 were studied by Gross (124). These investigations showed that T2 does not reproduce in washed and starved *E. coli* cells unless hydrolyzed casein or a mixture of amino acids is added to the medium. Isoleucine was an absolute requirement for phage synthesis when tested in amino acid mixtures. The successive deletion of many single amino acids from the mixture had no effect on phage synthesis.

Harding & Shive (125) reported that cyclopentaneglycine competitively inhibited the utilization of isoleucine by *E. coli* and other microorganism.

Reversal of toxicity was effected by either isoleucine or  $\alpha$ -keto- $\beta$ -methylvaleric acid. Low concentrations (10  $\mu$ g.) of the keto-acid exert a synergistic effect in the presence of higher concentrations of isoleucine (10–30  $\mu$ g.) in reversing the inhibitory effect of the analogue.

Reed *et al.* (126) studied the biosynthesis of leucine by baker's yeast grown on carboxy-labeled acetate. An examination of the label in the leucine skeleton revealed that there was almost exact duplication of the isotope pattern in carbons 1,2,3 and 4 with that of carbons, 5,4,3 and 2 of glutamic acid; the authors postulate two "acetate" condensations with  $\alpha$ -ketoglutaric acid to yield the leucine skeleton with a subsequent shift of hydroxyl from C<sup>4</sup> to C<sup>3</sup>.

Cohen & Hirsch (127) observed that cell suspensions of *E. coli*, supplemented with phosphate and glucose, synthesized L-threonine from DL-homoserine under aerobic conditions. Other wild type strains of *E. coli* also showed this threonine synthase activity. However, mutants which require threonine do not synthesize this amino acid from homoserine. The transformation of homoserine to threonine probably involves a shift of the hydroxyl group of homoserine to the  $\beta$ -position through an initial dehydration to the  $\beta,\gamma$ -unsaturated amino acid and a rehydration to threonine, as suggested by Teas *et al.* (128). The requirements of the organism for phosphate, glucose, and oxygen suggest that an intermediate of homoserine, such as the acyl phosphate of homoserine, may undergo this rearrangement.

Many of the biochemical steps in the aspartic acid, homoserine, threonine interrelationship (129) have been worked out by Black and co-workers (130, 131, 132) using cell-free extracts of baker's yeast.

*Dicarboxylic amino acids.*—McClure *et al.* (133) investigated the aspartic acid-lysine interrelationships in the nutrition of *Streptococcus faecalis* (6057). Under specific conditions lysine was required for growth, but the addition of aspartic acid in the presence of the B-vitamins, inositol and choline hydrochloride resulted in growth of the organism in the absence of exogenous lysine. The ability of aspartic acid to serve as a lysine precursor was further demonstrated by growing the organism in the presence of C<sup>14</sup> uniformly labeled aspartic acid. The lysine isolated from acid hydrolysates of these cells contained C<sup>14</sup>.

Ravel *et al.* (134) showed that, in the absence of biotin, *Lactobacillus arabinosus* requires aspartic acid. Lysine or threonine decreased the aspartic acid requirement by half, and a combination of lysine and threonine had a greater sparing effect than either metabolite alone. Neither  $\alpha$ - $\epsilon$ -diaminopimelic acid or  $\alpha$ -amino adipic acid replaced lysine in the nutrition of *L. arabinosus*, but the former of these compounds replaced lysine in the nutrition of *E. coli* (135, 136) and the latter in the nutrition of *Neurospora* mutants (137, 138).

Wahba *et al.* (139, 140) showed the interrelationships of aspartic acid to the biosynthesis of threonine, lysine, uracil, and purines. In the presence of cysteic acid, which inhibits the utilization of aspartic acid, the mixture

of metabolites showed greater degree of reversal of the inhibition than any single metabolite.

Ravel *et al.* (141) showed that glutamic acid competitively inhibited the utilization of aspartic acid in threonine biosynthesis by *Leuconostoc dextranicum* (8086) which required either aspartic acid or threonine for growth. Low concentrations of threonine prevent the toxicity of glutamic acid. Since oxalacetate or its precursors prevent the glutamic acid inhibition in a noncompetitive manner, it would appear that free aspartic acid is not formed from oxalacetate since the formation of free aspartic acid would reverse the glutamate inhibition competitively. Silverman, Romine & Daft (142) determined that L-isoglutamine can fulfill the glutamic acid requirement of *L. arabinosus* on prolonged incubation.

*Basic amino acids.*—The metabolism of lysine in lysineless mutants of *Neurospora* was studied by Schweet *et al.* (143). When these mutants were grown on labeled lysine, two labeled compounds were found to be present in the nonprotein fraction. These were pipecolic acid and a compound which yields  $\alpha$ -hydroxy-aminocaproic acid after hydrolysis. The latter compound is thought to be an intermediate which cyclizes to  $\Delta^1$  dehydropipecolic acid before reduction to pipecolic acid. Labeled  $\alpha$ -amino adipic acid was not formed by the tagged lysine. These data indicate that the synthetic pathway in the conversion of  $\alpha$ -amino-adipic acid to lysine by the mutant differs from the catabolic pathways.

The amino acid metabolism of an anaerobic organism isolated by soil enrichment technique was investigated by Stadtman (144) and Stadtman & White (145). This organism requires serine, threonine, proline, lysine, glycine, and arginine. Lysine, arginine, and proline were almost completely utilized. Utilization of serine, threonine, and glycine were less marked. It was determined that arginine plus proline or arginine plus lysine were effective substrates. Citrulline or ornithine would replace arginine. A peptide, supplied as an insulin hydrolysate, was also required for growth. Fermentation balance studies revealed that 5-aminovaleric acid was produced in amounts equivalent to 90 per cent of the proline that disappeared. The use of isotopically labeled ornithine revealed that the organism degrades the amino acid oxidatively to acetate, ammonia, and carbon dioxide. There was very little reductive deamination of ornithine. Lysine-2- $C^{14}$  was decomposed to acetate-2- $C^{14}$ , butyrate, and ammonia.

Ory *et al.* (146) investigated the role of glutamine in the biosynthesis of arginine by *L. arabinosus*. The organism cannot synthesize arginine in the absence of glutamine; citrulline is more effective than glutamine in replacing the arginine requirement (147). The labeled arginine was isolated from the hydrolyzed cells which were grown in the presence of  $N^{15}$ -glutamine. Studies on the distribution of the  $N^{15}$ -labeled arginine showed that the amide group of glutamine is the source of one of the nitrogen atoms of the guanido group. The results indicate that one of the essential functions of glutamine is to supply nitrogen for the conversion of ornithine to citrulline.

The utilization of benzoylamino acids by four species of bacteria was

studied by Itschner *et al.* (148). Most benzoylamino acids are not generally utilized by bacteria. However, bz-arginine is utilized by *Lactobacillus brevis* more effectively than arginine. Bz-leucine is used only by *L. arabinosus* and bz-methionine is utilized by *Streptococcus faecalis*. Hippuric acid and diBz-cystine were utilized by *Leuconostoc mesenteroides*. Hydrolysis to the free amino acid is probably necessary, before a benzoylamino acid is metabolized. An enzyme preparation obtained from *L. arabinosus* hydrolyzed bz-leucine much more rapidly than other benzoylamino acids.

Luzzati (149) studied the growth requirements of *E. coli* mutant W-74 which required either histidine or purine for growth. Histidine at a level of  $10^{-4}$ M was a more effective growth stimulant than any purine. Adenine and hypoxanthine promoted growth at lower concentrations but, at levels in excess of  $10^{-3}$ M, inhibited the organism. There was no synergistic effect upon adding purine plus histidine. Histidine could be replaced by histidinol or 5-imidazolelactic acid. Therefore, it appeared that histidine was the primary requirement for the mutant. An interrelationship between histidine and purines was earlier noted for *L. arabinosus* by Broquist & Snell (150).

Volcani *et al.* (151) studied the inhibitory effect of substituted anthranilic acids on the growth of *E. coli*. The inhibition of growth caused by 5-methylanthranilic acid, 5- and 4-fluoroanthranilic acids was reversed by anthranilic acid, indole, L-tryptophan, DL-phenylalanine, L-tyrosine,  $\alpha$ -ketoglutaric acid, L-glutamic acid, L-proline, L-hydroxyproline, and DL-methionone. Purines were found to reverse only the inhibition by the 5-methylanthranilic acid. These results indicated that the pathway of tryptophan synthesis involves the formation of indole from anthranilic acid as suggested by Rydon (152). The fact that a number of compounds not structurally related to anthranilic acid can relieve the inhibition caused by the analogues indicates either: (a) attack at more than one point in the metabolic chain, or (b) secondary disturbances after the primary attack.

Beresteher (153) studied the tryptophan requirements of eight microorganisms in the presence and absence of the inhibitor 5-methyltryptophan. With some of those organisms which required tryptophan for growth, the addition of the inhibitor increased the tryptophan or indole or anthranilic acid requirement. However, *Streptococcus lactis* (ATCC 8039), which required tryptophan specifically, was unaffected by the inhibitor. Skatole, tryptamine, indoleacetic acid, nicotinic acid, and indolepropionic acid could not substitute for the tryptophan requirement of any organism studied. Detailed studies with *Escherichia coli* and *Lactobacillus plantarum* (ATCC 8292) showed that the action of the inhibitor was reversed non-competitively by L-tryptophan or indole. Phenylalanine, however, reversed the action of 5-methyltryptophan competitively over a broad range of concentrations. Anthranilic acid reversed the inhibition of *E. coli* and *L. plantarum* by the analogue in a competitive manner. Kynurenine had only a slight activity in reversing the inhibition caused by 5-methyltryptophan. These data suggest a role of phenylalanine in tryptophan synthesis.

*Sulfur amino acids.*—Cross & Woods (154) and Cross (155) studied



methionine biosynthesis with cell-free extracts of *E. coli* mutants and *Streptobacterium plantarum* 10S cells. The data indicate that formate can supply the methyl groups of methionine either via serine or by a pathway not involving either serine or glycine. These latter observations are in accord with those of Arnstein & Neuberger (156), obtained with rats, and with those of Berg (157), obtained with cell-free extracts of pigeon liver.

Cross & Woods (154) reported that a cell-free extract of a glycine or serine requiring mutant of *E. coli* could synthesize methionine in the presence of DL-homocysteine, DL-serine, ATP, HDP, pyridoxal, phosphate, magnesium ions, and phosphate buffer. Methionine synthesis could be increased about 50 per cent by the addition of a heated extract of *E. coli*. The activity of the boiled extract could not be replaced by either yeast extract, PABA, folic acid, tetrahydrofolic acid, leucovorin, DPN, or vitamin B<sub>12</sub>. Glycine was produced in quantities approximately equimolar with the methionine synthesized. These data are in accord with the suggestion of Gibson & Woods (158) that the over-all reaction involves the reductive transfer of the  $\beta$ -carbon of serine to homocysteine to yield methionine. In whole cells, however, formate neither replaced nor spared serine in the presence or absence of glycine. Cross (155) has reported that the methionine requirement of *Streptobacterium plantarum* 10S was satisfied by carbon dioxide plus homocysteine. Leucovorin would not replace carbon dioxide. When C<sup>14</sup>-carbon dioxide was used there was no labeled carbon in the methyl group. However, if C<sup>14</sup>-formate was used in place of carbon dioxide, the methionine contained the label in the methyl group, but there was no significant amount of label in the serine of the hydrolyzed cell. Several communications have appeared on metabolic interrelationship between methionine and thiomethyladenosine (5'-methylthioadenosine). Schwartz & Shapiro (159) showed that thio-methyladenosine supported the growth of methionineless mutant of *Aerobacter aerogenes*. The organism responds partially to homocysteine. The addition of dimethylthetin or dimethyl- $\beta$ -proprothetin does not increase the homocysteine growth response. It was determined by the use of thio-methyladenosine doubly labeled with S<sup>35</sup> and C<sup>14</sup>-methyl, that over 80 per cent of the sulfur and methyl group of methionine originated from the thiomethyladenosine by thiomethylation. Experiments with cell-free extracts indicate that both  $\beta$ -aminobutyric acid and thiomethyladenosine are concerned in the biosynthesis of methionine. There was much less methionine synthesis by these cell-free extracts in the presence of homocysteine. These results are in accord with the data on the growth of the mutant at the expense of these substrates. Smith & Schlenk (160) previously reported that methionine was the source of the thiomethyl group of thiomethyladenosine in yeast. Further studies (161) with yeasts grown in the presence of DL-ethionine showed the formation of 5'-ethylthioadenosine. The thioethyl group was shown to arise from ethionine by employing DL-ethionine-S<sup>35</sup> and DL-ethionine-C<sup>14</sup> labeled in the methyl carbon of the ethyl group. The authors point out that the inhibitory effect of ethionine in various biological



systems may be the result of its conversion to S-adenosylethionine or 5'-ethylthioadenosine.

McRorie *et al.* (162) isolated a heat labile compound from cabbage juice which was more effective than methionine in preventing toxicity of sulfonamides for *E. coli*. The compound was identified as S-methylmethionine-sulfonium salt (3-amino-3-carboxypropyldimethylsulfonium salt). Some organisms which require methionine do not respond to the methylsulfonium derivative. McRorie *et al.* (163) determined the microbiological activity of the methylsulfonium derivative of methionine. The inhibition of growth caused by the ethylsulfonium derivative of methionine was competitively reversed by the methylsulfonium derivative of methionine, but reversed non-competitively by methionine. The authors indicate that the sulfonium compound may have functions other than that of a direct methionine precursor. Certain strains of *E. coli*, *Lactobacillus arabinosus*, and *L. casei* utilized the methylsulfonium compound while *Streptococcus faecalis* R, *Leuconostoc mesenteroides* P-60, and other strains of *E. coli* did not respond to this compound as a methionine replacement.

The role of a 4-carbon precursor in the biosynthesis of methionine, as indicated by the results of Schwartz & Shapiro (159), was further substantiated by the data of Kalan & Ceithaml (165) and of Friedman (166). Kalan & Ceithaml isolated 4 groups of methionineless *E. coli* mutants. The first group utilized methionine; the second group utilized cystathionine or homocysteine; the third group utilized vitamin B<sub>12</sub>; and the fourth group utilized a variety of compounds such as alanine, homoserine,  $\alpha$ -aminobutyric acid, valine, and isoleucine, which probably supplied the four carbon fragment. The growth of the mutants of the fourth group was stimulated by vitamin B<sub>12</sub>, but the vitamin did not replace the amino acid requirement. Friedman (166) reported that a mutant of *E. coli*, B187, utilized  $\alpha$ - $\gamma$ -diaminobutyric acid, but not  $\beta$ - or  $\gamma$ -aminobutyric acid, in place of  $\alpha$ -aminobutyric acid, homocysteine or methionine.

*Miscellaneous.*—The amino acid requirements of penicillin-resistant and penicillin-sensitive strains of *Micrococcus pyogenes* were determined by Bondi *et al.* (167). The minimal amino acid requirements for growth were relatively well-defined. Each strain required not more than 10 amino acids, six of which were required by all six strains.

Kaplan & Stock (168) observed that wild type *E. coli*, growing on synthetic salts-glucose agar plates, was inhibited by azaserine. This inhibition could be reversed by tryptophan, phenylalanine, or tyrosine. Studies with *E. coli* mutants which required aromatic amino acids or precursors for growth indicated that azaserine inhibited: (a) condensation of indole and serine to form tryptophan, and (b) synthesis of tyrosine and phenylalanine.

Halvorson (169) found that the inhibitory effect of azaserine on enzyme synthesis by yeast cells was partially reversed by the single amino acids, isoleucine, norleucine, phenylalanine, tyrosine, methionine, and valine; leucine, however, caused complete reversal of the azaserine inhibition.

Teeri (170) found that some D-amino acids in relatively high concentrations increased the lag phase of various lactobacilli. In some cases the L-form also caused a moderate delay in the initiation of growth. The increase in lag phase with the D-isomer may be related to the time required for conversion of the D to the L form, utilized for growth. The inhibitory action of the L-isomer may be related to an amino acid imbalance.

Johnson & Mays (171) isolated a strain of *Shigella dysenteriae* which required proline for growth. The organism responded to from 50 to 400  $\mu$ g. of proline. Ramsey & Padron (172) reported that an increase in resistance of *Micrococcus pyogenes* var. *aureus* to chloramphenicol resulted in a change in the nutritional requirements of the organism. Resistant strains of the organism showed decreased requirements for thiamin and niacin. The parent strain required cystine, proline, valine, and arginine for optimum growth; the resistant strain did not require arginine.

Wright & Cresson (173) described a convenient procedure whereby diaminopimelic acid may be readily isolated from the culture filtrate of an *E. coli* mutant.

#### PEPTIDES AND STREPOGENIN

More information has appeared to show that, under certain conditions, peptides may be more efficiently utilized than parent amino acids. Sirny, Wold & Schmidt (174) found that peptide-bound glycine gives a greater growth response than free glycine with *Leuconostoc mesenteroides* P-60. The glycine requirement for this organism can be modified by the presence of other amino acids. Thus, increasing amounts of arginine decrease the requirements for glycine. The synthesis of serine from glycine is partially inhibited by large amounts of alanine. This partial inhibition by alanine can be reversed by additional quantities of glycine or serine, and the peptides of glycine or serine are more effective than the free amino acids.

Woolley & Merrifield (175) have isolated peptides from insulin hydrolysates with high strepogenin activity. These peptides behave as homogeneous compounds during countercurrent extraction and ion-exchange chromatography. The most potent of these gave half maximum activity at 0.1  $\gamma$ /ml., and was 780 times as potent as the standard liver extract. It contained cystine, glutamic acid, glycine, serine, valine, and the leucines. Other peptides were obtained, varying in potency from 100 to 680. Their amino acid content was qualitatively different. Synthetic oxytocin (176), an octapeptide containing one equivalent of leucine, isoleucine, tryosine, proline, glutamic acid, aspartic acid, glycine, and cystine, was reported by Woolley & Merrifield (175) to have a strepogenin potency of 300. This peptide represents the first synthetic material with a high degree of activity. Oxytocin differs from the peptide obtained from insulin in that the latter contains serine and alanine and is devoid of tyrosine, proline, and aspartic acid. Arginine vasopressin (177) (free of oxytocin) had a strepogenin potency of 150. Several of the smaller synthetic peptides representing sequences in

oxytocin or vasopressin were inert. These results show that strepogenin activity does not reside in any specific amino acid configuration; furthermore, they establish that the activity is truly due to the peptide itself, since the potency of synthetic oxytocin dispels the probability of a highly potent impurity being associated with the peptides isolated from an insulin hydrollysate.

Roine, Gyllenberg & Salakivi (178) proposed an assay for strepogenin using *Lactobacillus bifidus*. The basal medium contained lactose, acetate and inorganic salts, and was supplemented with cysteine, pantothenic acid, and biotin. However, since a rigorous study of the nutritional requirements of the organism was not made and the strepogenin supplements used were not free of other accessory factors, it is not certain whether strepogenin was the only factor producing a response under the conditions described.

*Protein and peptide requirements of protozoa.*—Kidder, Dewey & Fuller (179) have shown that the nitrogen requirements of three ciliated protozoa differ mainly with respect to molecular size. *Tetrahymena* grows well on free amino acids. *Glaucoma scintillans* is dependent on polypeptides, but is able to utilize pure amino acids in the presence of suboptimal amounts of protein (casein) or polypeptide. Strepogenin from casein hydrolysates was ineffective. *Colpidium campylum* requires an intact protein (casein), which cannot be replaced by proteose-peptone. Molecular size is apparently important, since casein cannot be replaced by the smaller protein albumin. The authors make the interesting suggestion that the basis for these differences resides in the stimulation of the "swallowing response" (food vacuole formation). *Tetrahymena* responds with vacuole formation to substances in solution (drinking response). *Glaucoma* responds to polypeptides and small proteins, while *Colpidium* responds with vacuole formation only to large proteins. In the latter organism it is obviously impossible to investigate the specific amino acid requirements. With *Glaucoma*, however, this becomes possible in the presence of small amounts of polypeptides, which permits growth response to amino acid mixtures. By this procedure it was possible to show that *Glaucoma* had much the same pattern of amino acid requirements as *Tetrahymena*. A marked difference in the vitamin requirements existed. *Glaucoma* and *Colpidium* required approximately 20 times as much pantothenic acid as *Tetrahymena*.

Gjessing (180), continuing the investigations on growth stimulatory factors for *Staphylococcus albus* and *Streptococcus faecalis*, isolated acid-labile peptides from plasma dialysates which stimulated the growth of *Streptococcus faecalis*. One peptide was active at a level of less than 1.0  $\mu$ g.

#### UNKNOWN AND MISCELLANEOUS GROWTH FACTORS

The unique presence of *Lactobacillus bifidus* in the intestinal flora of breast-fed infants has stimulated research on the nutritive requirements of this organism (181). A particular variant, *L. bifidus* var. *Penn*, requires a growth factor that is present in human milk in 50 times the amount con-

tained in cow's milk. Tomarelli *et al.* reported (182) that this growth factor was also present in human saliva, salivary mucin, and hog gastric mucin, of which the latter was the richest source. The active factor in saliva, pepsin and hog gastric mucin, could not be separated from the mucin by extraction or dialysis. Mild acid hydrolysis, which resulted in a 35 per cent loss in activity, converted the activity into a dialyzable form. From this hydrolysate a crystalline disaccharide was obtained which was approximately twice as active as hog gastric mucin and 50 times as active as the solids of skimmed human milk. This disaccharide of hydrolysis yielded acetylglucosamine and galactose. D-glucosamine and N-acetylglucosamine were 10 percent as active as the disaccharide while D-galactosamine was inactive. The factor could also be replaced by large amounts of ammonium salts.

György *et al.* studied (183) the distribution of this factor in a variety of natural materials, finding it to be present in large amounts in the colostrum of many species, in saliva, and in meconium, which is present in the sterile intestinal lumen of the newborn infant. Approximately 40 to 75 per cent of the active factor in human milk is dialyzable (184). By the use of chromatography on paper and charcoal, four different active components were obtained from human milk. They yielded on hydrolysis: acetic acid, glucosamine, L-fucose, D-glucose, and D-galactose. All contained N-acetylglucosamine; one was lacking in fucose.  $\beta$ -methyl-N-acetyl-D-glucosaminide was observed by Rose, Kuhn, Zilliken & György (185) to be approximately 35 per cent as active as the disaccharide of galactose and N-acetyl-D-glucosamine. The corresponding  $\alpha$ -methyl-N-acetyl-D-glucosaminide was completely inactive by itself although it did markedly augment the activity of small amounts of the  $\beta$ -methyl derivative. N-acetylglucosamine,  $\alpha$ - or  $\beta$ -methylglucosides were inactive in potentiating the action of  $\beta$ -methyl-N-acetyl-D-glucosaminide. An enzyme present in *L. bifidus* var. *Penn* was capable of splitting the  $\beta$ -methyl derivative but not the  $\alpha$ -form.

In a more detailed study with this enzyme, Zilliken *et al.* (186) obtained a cell-free enzyme preparation from *L. bifidus* which hydrolyzed these growth factors with liberation of N-acetylglucosamine. This enzyme, when permitted to act on a mixture of lactose and N-acetylglucosamine, increased the biological activity of the mixture three-fold. Two isomeric disaccharides were formed, one of which was identical with the galactose-N-acetylglucosamine isolated from hog mucosa. The other isomer was relatively inactive biologically. This enzyme which inactivates the *bifidus* factor was found by György, Rose & Springer (187) to be present in the strain of *L. bifidus* requiring the factor, but not in other strains which do not require the factor. This enzyme is also present in saliva and in culture filtrates of *Cl. welchii*. It inactivates both blood group activity and the *bifidus* factor.

A growth factor for an avian strain of *Lactobacillus bifidus* has been investigated by Shorb & Veltre (188). The disaccharide, galactose-n-acetylglucosamine, which is essential for growth of certain human *L. bifidus*

variants (182) proved inactive for the avian strain. The factor was insoluble in fat solvents, soluble in ethanol and water, and adsorbable on norit.

Several unidentified growth factors have been described as being essential or stimulatory to the growth of various organisms. Rege & Sreenivasan (190) reported that the early growth of *L. casei* was increased by various natural materials such as yeast extract, peptone, and a fermentation product of *Aerobacter aerogenes*. The adsorbability on charcoal and solubility in 95 per cent ethanol were cited as evidence of nonidentity with strepogenin. However, the activity of a trypsin digest of casein suggests that part of the observed response may be due to strepogenin, and until vitamin-free preparations of strepogenin have been tested, judgment must be reserved on the nonidentity of this new factor with known compounds.

The nutritional requirements of three phagotrophic slime molds were studied by Sussman & Bradley (191). These organisms, *Dictyostelium discoideum*, *D. mucoroides* and *D. purpurem*, which resemble the soil amoebae, can be grown symbiotically with *Aerobacter aerogenes* or *E. coli*. The bacterial associates could be replaced by a protein fraction, isolated by repeated precipitation of the ground cells of *Aerobacter aerogenes*. The protein fraction, which amounted to 6 per cent of the weight of the bacterial cell, appeared homogeneous by sedimentation patterns, and had a molecular weight between 25,000 and 40,000. This protein was found to be very labile at pH 9.5 and pH 3.5 but could be autoclaved for 15 min. at pH 7.0, at which hydrogen ion concentration it is insoluble, without loss of activity. It was inactivated by trypsin; and fractions of protein from cells which had been cytolysed by glucose or toluene treatment rather than by grinding proved to be inactive. The protein is required in relatively large amount, and apparently can serve as an energy source in the absence of a carbohydrate source in the medium. Autoclaved pastes prepared from two gram-positive organisms were biologically active, but the protein had solubility properties different from those of the proteins obtained from *A. aerogenes* and two other gram-negative organisms. Glutenin from wheat, which has very similar solubility properties to the protein from *A. aerogenes*, was completely inactive.

An unidentified growth factor for *Streptococcus faecalis* 8041 has been postulated by Hill (192). This factor, which is present in yeast extract, liver, and pancreas is stimulatory during the early growth period.

Rogers, King & Cheldelin (193) found that N-glucosylglycine and reaction products between glycine and carbohydrates stimulate the growth of *Lactobacillus gayoni* 8289. Initiation of growth of this organism is delayed when the medium is sterilized by filtration rather than by autoclaving. Glycosyl compounds are formed by the browning reaction between amino acids and reducing sugars during autoclaving. Glycine was the only amino acid which would yield active preparations when heated with glucose. A number of reducing sugars and simpler ketones and aldehydes such as

glyceraldehyde, dihydroxyacetone, and methylglyoxal reacted with glycine to give active products.

The nutritional requirements of the Donovan organism (*Donovania granulomatis*) were studied by Hall, Dienst & Chen (194). This bacterium can be cultivated on a medium containing peptone, glucose, and a sterile suspension of fresh egg yolk. The egg yolk could be replaced by a nondiffusible fraction of egg yolk, whose activity was destroyed by acid or alkaline hydrolysis or by digestion with trypsin. The major portion of the activity resided in the vitellin fraction of the protein. Other protein sources such as beef serum and chicken liver were inactive.

Fisher (195) has reported hemin to be a growth factor for isoniazid-resistant variants of *Mycobacterium tuberculosis* strain H37R. The development of resistance to isoniazid in a "one-step" culture method occurred only in the presence of hemin-containing materials. Hemin also reversed isoniazid inhibition of the growth of *M. tuberculosis*. It thus appears that isoniazid interferes with the synthesis of hemin or related compounds by this organism.

Burton, Sowden & Lockhead (196) found that *Arthrobacter terregens* required a factor which is present in soil, and which is synthesized by *Arthrobacter pascens*. This "terregens factor" was obtained in the form of a highly active concentrate from *A. pascens* fermentation broth. This concentrate was active at a concentration of .001  $\mu\text{g./ml}$ . Although the terregens factor contains only traces of iron, it combines with ferrous or ferric ion to form reddish-brown complexes. Crystallization of this factor has not been achieved. The most highly active concentrates which were prepared contained a number of amino acids, and were free of sugars, purines, and pyrimidines. Activity for *A. terregens* was also shown by iron-containing complexes such as hemin, ferrichrome (197), and coprogen (198).

#### MINERALS

Sirny *et al.* (199) investigated the effect of potassium and sodium on the vitamin and amino acid requirements of various assay organisms. In most cases the use of a low sodium, high potassium medium resulted in greater acid production. With pantothenic acid, however, the requirement for this vitamin was reduced by the addition of sodium to the medium.

Friedman & Fox (200) studied the effect of potassium on growth, and on utilization of glucose, ammonia, and phosphate by different strains of *E. coli*. Optimal growth was obtained at potassium concentrations of .0026 to .04 molar. The uptake of glucose, ammonia, and phosphate was decreased on potassium-deficient media. At intermediate levels of potassium, increasing amounts of sodium or magnesium were inhibitory, but the inhibition could not be reversed by increasing amounts of potassium. Increasing the amount of potassium produced a greater increase in purine synthesis, as measured by accumulation of 4-amino-5-imidazolecarboxamide, than in growth.



Potassium concentrations between .0045 and .050 M were optimal for the growth of *Micrococcus pyogenes* [Haynes, Kuehne & Rhodes (201)]. Concentrations of less than .0045 M would not permit maximum growth, and concentrations higher than .050 M were inhibitory.

Hendlin & Wall (202) reported that the toxicity of large amounts of sodium chloride for *Lactobacillus leichmannii* could be reversed within certain limits by vitamin B<sub>12</sub>. With *Lactobacillus lactis* Dorner, which also requires vitamin B<sub>12</sub>, sodium chloride was toxic but vitamin B<sub>12</sub> had no reversing effect.

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## METABOLISM OF MICROORGANISMS<sup>1,2</sup>

BY E. A. DELWICHE

*Laboratory of Bacteriology, College of Agriculture, Cornell  
University, Ithaca, New York*

Because of space limitation, it is impossible to review adequately all of the current literature (1954) within the purview of the title. Certain exclusions have therefore been made, based upon the opinion that the effort should not include a review of reviews. Hence, those topics reviewed within the year, and those in press at the time of writing are largely excluded. We take notice in this regard to "Bioluminescence" (1), "The Stickland Reaction" (2), "Bacterial Cytochromes" (3), "The Preparation of Cell-free Enzymes from Microorganisms" (4), "Oxidation and Evolution of Molecular Hydrogen by Microorganisms" (5), and those sections of the review on carbohydrate metabolism in microorganisms by Gunsalus, Horecker & Wood (6) that deal with the hexose monophosphate pathway. In addition two excellent reviews and one published symposium not generally restricted to microbiological activity, yet pertinent to the subject, are called to the attention of the reader. Much that is contained in Ochoa's "Enzymic Mechanisms in the Citric Acid Cycle" (7), Racker's "Alternate Pathways of Glucose and Fructose Metabolism" (8), and in the Johns Hopkins University "Symposium on Amino Acid Metabolism" (9) bears directly upon the subject at hand.

This author is of the opinion that no review can be wholly objective, for the very choice of subject matter is dictated, at least to some degree, by personal interest. He thus begs the forbearance of those contributors whose work was overlooked or otherwise neglected.

### ENZYMES

Continued interest is apparent in isomeric specificities displayed by bacterial enzyme systems. Stanier & Ingraham (10) and MacDonald, Stanier & Ingraham (11), in the isolation and purification of protocatechuic acid oxidase from *Pseudomonas fluorescens*, isolated the intermediate product in the oxidative conversion of protocatechuic acid to  $\beta$ -ketoadipic acid

<sup>1</sup> The literature survey for this review was concluded in December, 1954.

<sup>2</sup> The following abbreviations are used: AMP for adenosinemonophosphate; ADP for adenosinediphosphate; ATP for adenosinetriphosphate; DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; TPN for triphosphopyridine nucleotide; TPNH for reduced triphosphopyridinenucleotide; FAD for flavin adenine dinucleotide; FMN for flavin mononucleotide; DPT for diphosphothiamin; RNA for ribonucleic acid; DNA for desoxyribonucleic acid; Co A for Coenzyme A; PA for pantothenic acid; PABA for *para*-aminobenzoic acid; ITP for inosinetriphosphate.

and identified it as one of the  $\beta$ -carboxymuconic acids. On the basis of certain of its chemical properties it was suggested that the molecule contained at least one *cis* double bond and was probably the *cis-cis* isomer. The purified oxidase, which was separated from the decarboxylating system, was specific for the oxidation of protocatechuic acid. The purified enzyme was found to require no demonstrable cofactors and in this respect is distinguishable from the functionally similar pyrocatecase and homogentisic acid oxidase (10). Separate and distinct enzymes convert *cis-cis* muconic acid via (+)- $\gamma$ -carboxymethyl- $\Delta^{\alpha}$ -butenolide to  $\beta$ -keto adipic acid. The two metabolic paths converge at or just preceding the  $\beta$ -keto adipate level of degradation. The immediate product of catechol oxidation [Sistrom & Stanier (12)] in *P. fluorescens* was identified as *cis-cis* muconic acid. The conversion of *cis-cis* muconic acid to  $\beta$ -keto adipic acid was found to involve a lactonizing step to the butenolide and a delactonizing conversion to  $\beta$ -keto adipic acid. An exhaustive study of the lactonizing enzyme [Sistrom & Stanier (13)] revealed that both the *cis-cis* and the *cis-trans* isomers were vulnerable to lactonization, the *cis-cis* being 95 per cent converted at equilibrium (pH

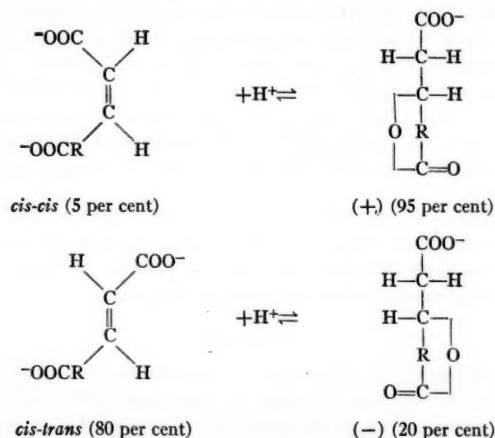


FIG. 1. The two reversible reactions catalyzed by the lactonizing enzyme [Sistrom & Stanier (13)].

8.0), while the equilibrium of the *cis-trans* isomer was 20 per cent in the direction of the lactone (see Fig. 1). The delactonizing enzyme was inactive on the unnatural (-)-lactone. According to Sistrom & Stanier (13) these equilibrium data are compatible with the energy changes recorded for similar isomerizations. The apparent lack of enzyme specificity seems very adequately explained by imposing the "three-point attachment" theory. In the forward reaction (Fig. 1) only one bond of the  $\gamma$ - $\delta$  double bond is split and in the reverse reaction only one of the  $\delta$ -hydrogens is removed. The result is the



conversion by one enzyme of two geometrical isomers into optical enantiomorphs. Interconversion of the two geometrical isomers or of the two enantiomorphs cannot occur.

The claim of Schwartz & Carter (14) for a non-enzymatic case of "citric acid type" asymmetry seems likely to arouse controversy. Based upon a study of  $\beta$ -phenylglutaric anhydride cleavage with optically active amines, which yielded unequal amounts of two isomers, these investigators propose that under certain structural conditions a molecule, such as of the type depicted in Figure 2, can display "citric acid type" asymmetry in the ab-

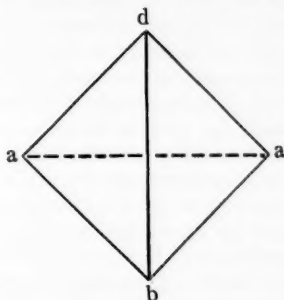


FIG. 2. "Meso" carbon atom [Schwartz & Carter (14)].

sence of an enzyme. Such a substituted carbon is designated by them as a "meso" carbon atom. Wolfrom (15), however, has pointed out that in the case under study a new asymmetric center has been created; and, the reaction of 3-phenylglutaric anhydride with (*levo*)- $\alpha$ -methylbenzylamine has resulted in an equilibrium reaction with the products being two diastereoisomers possessing different chemical energies. The explanation that follows, according to Wolfrom, is that a disproportionate ratio of products results as a consequence of the different equilibria of the two reactions.

Swick & Nakao (16) have extended Ogston's principles to glycerol, a molecule with the same degree of symmetry as citric acid. Glycerol-1- $C^{14}$  isolated from a yeast fermentation of glucose-3,4- $C^{14}$  was fed to rats. The glucose derived from the liver glycogen contained 91 per cent of the radioactivity in the 3 and 4 positions, and the lactic acid formed from it by a *Lactobacillus casei* fermentation contained a similar proportion of radioactivity in the carboxyl groups. Thus they concluded that glycerol, like citric acid, can be regarded as a biologically asymmetrical compound. Schambye, Wood & Popják (17) in a simultaneous publication conclude similarly after conducting somewhat parallel experiments, the labeled glycerol, however, being biosynthesized by feeding acetate-1- $C^{14}$  to goats, and the final glucose being degraded by fermentation with *Leuconostoc mesenteroides*. Bublitz & Kennedy (18, 19) isolated and purified a glycerokinase from rat liver. The asymmetrically labeled glycerol furnished by

Swick & Nakao (16) was enzymatically phosphorylated, and by chemical degradation they established that the unphosphorylated primary hydroxyl contained 83 per cent of the total radioactivity. Thus excellent experimental evidence is brought to bear in support of the suggestion by Schambye, Wood & Popják (17) that the phosphorylation of glycerol is the primary step in the asymmetrical metabolism of the compound.

In the field of adaptive enzymes it is becoming increasingly more apparent that induced enzyme synthesis is not accomplished through the simple modification of gross precursor molecules. Rotman & Spiegelman (20), for example, found that with  $\beta$ -galactosidase synthesis in *Escherichia coli* cells uniformly labeled with  $C^{14}$ , less than 1 per cent of the purified enzyme was derived from components existing prior to the addition of inducer material. Their results necessitate the conclusion that induced enzyme synthesis occurs virtually completely through the utilization of free amino acids. The data they present further indicates that protein synthesis in general is an irreversible process. Likewise subject to critical scrutiny are the concepts of inducer molecules acting as complexing agents. By measuring and comparing the dissociation constants of  $\alpha$ -methyl glucoside as an inducer and as a complexant with maltose, Spiegelman & Halvorson (21) have ascertained that with *Saccharomyces cerevisiae*, the glucoside does not combine with maltase during the induction process.

Sher & Malette (22) reported on the adaptive formation of lysine decarboxylase by *E. coli* B occurring in the absence of cell division, thus eliminating selection as the causative factor in the development of the enzyme. Their basic observations were the appearance and disappearance of the enzyme in one and one-half generations, and, through the use of sulfur mustard to block cell division, the formation of the enzyme in a completely non-multiplying culture. In a second report (23) they describe the properties of the purified lysine decarboxylase.

Sheffner & McClary (24) contend that the onset and degree of the aerobic galactose fermentation in yeast during adaptation is dependent upon the preliminary accumulation of reserves and intermediates, and that the accumulation of fructose phosphates during the oxidation of galactose indicates that the oxidation proceeds to some extent through the Emden-Meyerhof scheme. As a consequence of their findings they pointed out possible errors in the use of whole cell fermentation experiments in attempting to demonstrate the autocatalytic nature of adaptive enzyme synthesis.

Moat & Lichstein (25) stimulated adaptation to sucrose fermentation in biotin-deficient cells of *S. cerevisiae* by the addition of biotin and concluded that this vitamin may be concerned in the synthesis of enzymes concerned in the sucrose fermentation. Chambers & Delwiche (26) concluded similarly that biotin may have a function in apoenzyme formation.

The work of Dagley (27) further describes factors which are of importance in the interpretation of adaptive enzyme experiments. In *Aerobacter aerogenes* and in *E. coli* citridesmolase activity was found to be very markedly

influenced by the concentration of the carbon source. Cell permeability factors also complicated the picture. Bernheim (28) noted an effect of quinones on adaptive enzyme formation in a strain of *Pseudomonas aeruginosa*. Landeman (29), seeing a need for knowing the characteristics of  $\beta$ -galactosidase formation in *Neurospora crassa*, reported in detail on his investigation of strain Emerson 5256A. Chantrenne & Courtois (30) find that catalase production is induced by aeration.

The publication of Williams & Wilson (31), dealing with the equilibration of succinate solutions with adapted and unadapted cells of *Azotobacter vinelandii*, bears excellent evidence that adaptation to a particular substrate may not involve specific substrate adaptive enzyme formation, selection, nor even simple permeability. Both types of cells were found to remove equal amounts of succinate when exposed to succinate solutions, and both types of cells yielded cell-free succinoxidase activity of approximately the same magnitude. They interpret these findings as changes connected with an active transport process associated with the metabolism. Foulkes' (32) study of citrate metabolism in yeast pointed likewise to an assimilation process involving more than mere cell wall permeability. Cells disrupted by freezing absorbed but did not metabolize citrate. Treatment with chloroform enabled the preparation to both absorb and metabolize citrate. Volk (33) visualizes that the fluoride-resistant fermentation by *Propionibacterium* involves a decreased permeability to NaF, and that growth in the presence of NaF inhibits the phosphatases of the cell membrane so as to eliminate or decrease to a negligible rate the fermentation of phosphorylated intermediates. His observations are of particular interest in that they provide an explanation for the long-recognized fluoride-insensitive glucose fermentation by *P. pentosaceum* cultures which cannot utilize Emden-Meyerhof phosphorylated intermediates.

Spores are now being studied at the biochemical and enzyme level. Lawrence & Halvorson (34) succeeded in demonstrating catalase activity in "well-cleaned" spores of *Bacillus terminalis* (*Bacillus cereus*). The activity was found to be highly thermal resistant when compared to that of the vegetative cells. Grinding the spores caused an increase in specific activity but also changed the thermal inactivation data, causing the appearance of two different inactivation curves. These data along with the observation that fractions differing in heat resistance cannot be separated by centrifugation, caused these investigators to suggest that resistance is not solely a function of particle size, but rather that the heat sensitive fraction is contained within the spore and is not measurable until the spore is disrupted. A five-fold increase in catalase activity resulting from the grinding of *Bacillus globigii* spores is seen to strengthen this hypothesis. Similarly, a heat resistant alanine racemase from *B. terminalis* (*B. cereus*) spores was isolated [Stewart & Halvorson (35)]. In this case, however, the heat resistant fraction was separable by centrifugation or ammonium sulfate fractionation, and therefore could be associated with particle size. The particles

were found to be resistant to pepsin inactivation as well as heat denaturation. Further sonic oscillation resulted in solubilization with attendant increases in heat and pepsin susceptibility. Church, Halvorson & Halvorson (36) demonstrated the independence of spore germination and alanine racemase activity in six species of aerobic sporeformers, thus discounting the attractive hypothesis that alanine racemase and the D- to L- alanine inter-conversions are of direct significance in spore germination.

The spores of *B. megaterium* have been found to contain as much of the aspartic-glutamic transaminase as do the vegetative cells [Levinson & Sevag (37)]. This finding is in conflict with that of Hardwick & Foster (38). In the light of present studies it appears that the conditions of cell disruption employed by the former workers completely solubilize the enzyme, whereas the method of the latter group caused the enzyme to be discarded in the active or inactive state in the particulate fraction of the disrupted spore mass.

Germinating spores were found to produce a non-dialyzable peptide in the spore exudate [Strange & Powell (39)]. The peptide was characterized chemically as containing  $\alpha$ - $\epsilon$ -diamino pimelic acid, glutamic acid, alanine, acetyl derivatives of glucosamine, and an unidentified sugar amine. Record & Grinstead (40) determined some of its physico-chemical properties. The suggestion is made by Strange & Powell (39) that the peptide may be derived from the spore surface when the permeability of the structure is modified during germination or through mechanical damage.

Militzer and coworkers (41) continued their studies on the nature of thermophily. Their most recent report (41) described the heat lability of the pyruvic oxidase from the thermophile, *Bacillus stearothermophilus* No. 2184. Unlike their previously reported enzyme systems (42, 43), they found that this pyruvic oxidase was easily inactivated at the growth temperature of the organism unless magnesium ions, pyruvate, and oxygen were present. The concept of "soluble stabilizers" in addition to increased protein stability and more rapid protein replacement [Allen (44)] must now be imposed in attempting to elucidate the secrets of thermophily.

Sevag, Newcomb & Miller (45) prepared an anti- $\alpha$ -glycerol phosphatase and found that it was capable of inhibiting the activity of purified phosphatase preparations and also the activity of the intact yeast cells. It was suggested that the enzyme is located at the surface of the cell. Demis, Rothstein & Meier (46) reported that in the case of yeast invertase virtually 100 per cent of the added sucrose can be accounted for as extracellular sucrose or its hydrolytic products. They concluded that hydrolysis by surface bound invertase accounts for essentially all of the sucrose utilization by the cell.

Much information that deals with the isolation and properties of individual enzymes has appeared during the year. Oesper (47) has added to our knowledge of glyceraldehyde-3-phosphate dehydrogenase. Purified preparations from either yeast or muscle will catalyze the exchange of phos-

phate with the 1 position of 1,3 diphosphoglyceric acid in the absence of added DPN, hence the conclusion is made that the oxidative step precedes phosphorylation. Rafter, Chaykin & Krebs (48) described a curious reaction between DPNH and yeast or muscle triose phosphate dehydrogenase. The reaction was characterized by the loss of the absorption band at 340 m $\mu$  and an increased absorption in the 280 to 290 m $\mu$  region. In the words of the authors "there is no suggestion that this reaction is in any way connected with the mechanism of triose phosphate oxidation." In view of the extensive employment of spectrophotometric measurements of the pyridine nucleotides in analytical procedures, their admonishment of a necessity for an awareness of this DPNH modification seems particularly appropriate. DeMoss (49) described the purification and properties of a TPN-dependent alcohol dehydrogenase from *L. mesenteroides*. The aldolase from *Aspergillus niger* is sensitive to ethylenediamine tetraacetic acid and can be reactivated with a zinc ion [Jagannathan & Singh (50)].

Weimberg & Doudoroff (51) partially purified, studied, and compared the sucrose phosphorylases of *Pseudomonas saccharophila*, *Pseudomonas putrefaciens*, and *L. mesenteroides*. Only minor differences in their properties appeared to exist. All are transglucosidases—enzymes capable of preserving the  $\alpha$ -glucosidic configuration in the exchange between sucrose and glucose-1-phosphate. The *L. mesenteroides* enzyme was more highly purified and was found to possess no demonstrable cofactor. After extensive purification, hydrolase activity was still present, apparently being an intrinsic property of the enzyme.

Other bacterial enzymes which have been purified and studied include a urease from *Bacillus pasteurii* (52), a specific 5'-nucleotidase (53), and multiple microbial cellulases (54). In yeast specific studies have been reported on a nicotinamidase (55) and an alcohol dehydrogenase (56). The isolation of a formylkynurenine formamidase from *Neurospora* [Jakoby (57)] is offered as evidence that formyl kyneurinin is an intermediate in nicotinic acid synthesis in this organism.

#### CARBOHYDRATES

Interesting new advances have been recorded in the field of microbial cellulose synthesis. Minor and coworkers (58) studied the incorporation of D-glucose-1-C<sup>14</sup> into cell wall cellulose by *Acetobacter xylinum*. They observed that the cellulose units had a lower specific radioactivity than that of the glucose, and that the incorporations of the activity into the number 1 position of the hexose unit was 82 and 70 per cent depending upon whether ethanol was present or absent. The remainder of the radioactivity was found in the number 2 and number 3 positions. This is accordingly interpreted as meaning that polymerization of the D-glucose units directly is not the sole mechanism by which the cellulose is formed, and that some of the cellulose structure must have arisen from cleavage products. With D-mannitol-1-C<sup>14</sup> they noted (59) 84 to 96 per cent incorporation into positions 1 and 6 with

the residual activity in positions 2 and 5. Ethanol, although it increased the yield as well as the  $C^{14}$  content, did not affect the isotope distribution. In a later report from the same laboratory Greathouse, Shirk & Minor (60) submit that ethanol and acetate significantly enhanced cellulose synthesis although neither were incorporated into the cellulose structure. Hestrin & Schramm (61) described the preparation of cellulose-free non-proliferating cells of *A. xylinum* which polymerized glucose into high polymer cellulose. The absence of cellulose within the cells, the properties of the isolated and purified polymer, along with a consideration of the kinetics of its formation lead them to the consideration that some constituent on the outer surface of the cell is the terminal catalyst for cellulose production. These data favor the hypothesis expressed by Minor *et al.* (59) that cellulose formation, even that fraction which occurs via involved pathways, is primarily a terminal biosynthesis by direct polymerization.

Antigenicity changes can be caused to occur in the capsular substance of *Leuconostoc dextranicum* by the application of the method of McCarty (62) [Whiteside-Carlson, *et al.* (63)], but the derived organisms still produce dextran; hence, the material responsible for change in antigenicity was considered as residing in the non-dextran capsular material. No correlation between antigenic structure and nutritional requirements could be established by this work.

Working with yeast invertase preparations acting on sucrose, Bacon (64) identified by means of partition chromatography two disaccharide fractions and three trisaccharides. These data are presented in support of the hypothesis that yeast invertase functions by transfer of the fructose residue from sucrose to water or to another substance possessing a primary alcoholic group. Chung & Nickerson (65) approached the complexities of polysaccharide synthesis in yeast by studying fluoride inhibition and its reversal. It is contended by them that growth inhibition by fluoride is due to the inhibition of polysaccharide synthesis. The reversal of fluoride inhibition with glucose-1-phosphate is considered as evidence that the inhibition site is the enzyme phosphoglucomutase. In view of the fact that other hexose phosphates would not relieve the fluoride inhibition, this interpretation seems quite plausible. Mannose-1-phosphate is also concerned in polysaccharide synthesis, with an enzymatic interconversion with glucose-1-phosphate being implicated also by their findings.

A study of the utilization of 1- $C^{14}$ -D-glucose in the production of mannan by *Torula utilis* revealed that direct incorporation of the glucose into the mannan unit must not have occurred. Sowden *et al.* (66) degraded the mannan produced under these conditions, and record that the mannan was devoid of radioactivity. This observation is in contradistinction to the findings of Gilvarg (67) with *S. cerevisiae*, where the mannan was found to be produced without chain cleavage. Differences in extraction procedures lead the Sowden group to consider the interesting possibility that D-mannose

is present in yeast in two different types of combination and that different biosynthetic routes may exist for the different combinations. Their finding of only insignificant amounts of radioactivity in the nucleic acid ribose is in good agreement with the current concepts of pentose synthesis.

Hansen & Craine (68) succeeded in isolating and purifying the galactowaldenase from *Lactobacillus bulgaricus*. The separation from phosphoglucomutase enabled them to establish the equilibrium of the reaction as 73 to 79 per cent in the direction of the glucose ester. They noted that one of their preparations could convert galactose-1-phosphate to glucose-1-phosphate with the reverse reaction much slower than would be predicted from the equilibrium experiments they conducted. It was suggested that the enzymes may be multiple. Steele, White & Pierce (69), while studying capsular polysaccharide synthesis by Group A streptococci, made the surprising observation that a typically heterolactic type of fermentation obtains when galactose is the carbon source. Formic acid, acetic acid, and ethanol in a ratio of 2-1-1 accounted for approximately 50 per cent of the galactose fermented. These results are as yet quite uninterpretable insofar as metabolic pathways are concerned, and, as the workers suggest, may only represent a matter of degree when compared to the traces of heterolactic fermentation products produced during the typical homolactic fermentation.

With regard to pentose metabolism, much has been accomplished which shall not be covered in this review [See Gunsalus, Horecker & Wood (6)]. Two papers, somewhat isolated in their context, however, shall be briefly summarized. Hochster & Watson (70) reported the isolation of a xylose isomerase from *Pseudomonas hydrophila*. The preparation was specific for the conversion of xylose to xylulose. These investigators take note of the fact that the mechanism of synthesis of ribonucleosides in organisms such as *P. hydrophila* grown on xylose poses some interesting questions. The fermentation of L-arabinose-1-C<sup>14</sup> by *Propionibacterium pentosaceum* reported by Rappaport & Barker (71) resulted in the production of radioactivity in all of the carbons of the fermentation products, propionate, acetate, and carbon dioxide; although the methyl carbon of the acetate had the highest specific activity. In addition, the  $\alpha$  and  $\beta$  carbons of the propionate (which, according to the interpretation given, could originate from acetate via succinate through known biosynthetic reactions) were found to be more strongly labeled than the carboxyl carbon. The suggestion is made that the acetate was derived from carbons 1 and 2 of the arabinose. In the opinion of the reviewer, this work typifies the difficulties of interpretation encountered with whole cell isotope experiments involving propionic acid bacteria.

Wilner & Clifton (72) studied oxidative assimilation by *Bacillus subtilis* and noted the same general pattern of assimilation as obtained with other microorganisms. Data from manometric observations caused them to conclude that the endogenous respiration of the organism is not suppressed



during exogenous respiration. Kopper (73) followed substrate assimilation by measuring the subsequent reducing activity in 2,3,5-triphenyltetrazolium chloride.

From the type of papers published it would seem that the year 1954 marked the final acceptance of the concept of almost universal occurrence of the tricarboxylic acid cycle. Much of the work reported dealt with a consideration of the primary role of the cycle. Roberts *et al.* (74), for example, by observing the incorporation of  $C^{14}$  from various carbon sources, concluded that in the case of proliferating *E. coli* cultures, the cycle furnishes 50 per cent of the carbon required for protein synthesis, but is relatively unimportant for oxidations. Their method, in their opinion, permits them "to demonstrate the occurrence of most of the features predicted by the Krebs' cycle." McQuillen & Roberts (75), working with the same species, observed the fixation of radioactive acetate into various amino acid fractions of actively growing *E. coli*. Specific activity measurements of a number of isolated amino acids, as well as the results of competition experiments, were interpretable in terms of biosynthetic relationships involving the tricarboxylic acid cycle. They conclude, also, that the function in the actively growing *E. coli* cell is mainly one of synthesis. On the other hand Swim & Krampitz (76) and Saz & Krampitz (77) found that with resting cells of *E. coli* and with *Micrococcus lysodeikticus*, acetate is extensively oxidized by a citric acid cycle. These experiments, which were designed to test the validity of non-equilibration tests between isotopic substrate and added carrier as a criterion for the absence of the cycle, offer convincing evidence that the lack of incorporation of isotope into an added carrier is not conclusive proof against the occurrence of the compound as an intermediate. It has also been established by Swim & Krampitz (78) that methyl to methyl acetate condensation does not occur in *E. coli*. In the area of mold metabolism compliance with the principles of comparative biochemistry insofar as the citric acid cycle is concerned seems also assured. Casida & Knight (79) obtained evidence for the cycle in cell-free preparations of *Penicillium chrysogenum* after the employment of phosphate and fluoride as phosphatase inhibitors. Enzymatic adaptation is also an important factor in the interpretation of data for cyclic oxidation mechanisms. Williams & Wilson (80) describe studies of this sort with *A. vinelandii*, and Englesberg *et al.* (81, 82) studied the enzymatic changes accompanying the shift from anaerobiosis to aerobiosis in *Pasteurella pestis*.

#### ORGANIC ACID METABOLISM

Investigations concerning the metabolism of acetate and related fields continue to occupy the interests of many workers. Umbarger (83) tested the effect of varied environmental conditions on the activity of the acetate and oxalacetate condensation step in *E. coli*. The important factors which enhanced "citrogenase" activity were found to be oxygen, a source of acetate or an acetate-producing molecule as an inducer, and a need for metabolites

synthesized via pathways involving the condensation enzyme. Citrogenase activity was essentially absent in cells grown anaerobically on a casein hydrolysate, yeast extract, and glucose medium, although these cells were capable of subsequent adaptation. Rose *et al.* (84) purified the acetokinase of *E. coli*. The purified enzyme (100x) reacted specifically with acetate and to a very limited extent with other acids, requiring ATP and  $Mg^{++}$  or  $Mn^{++}$ . ITP could be substituted. Razzell & Gunsalus (85), in studies on the intricacies of acetate and acetyl-phosphate producing systems, reported that the Crookes strain of *E. coli* possesses a phosphate-independent acetate generating pyruvate system in addition to the dismutative acetyl-phosphate generating system (86). Strain X-19 of *Proteus vulgaris* was found to have an acetyl phosphate system as well as the acetate generating, oxygen consuming system (87, 88). Fractional centrifugation of the *E. coli* extracts yielded a particulate hydrogen transport fraction and a soluble DPT requiring carboxylase which would not link with oxygen. The fractions could be combined to give acetate production with concomitant oxygen consumption, and were mutually replaceable with the *Proteus* fractions of Moyed & O'Kane (88). Application of the fractionation methods for the *E. coli* phosphate generating [Hager *et al.* (89)] system yielded analogous enzyme fractions. The *Proteus* carboxylase does not contain lipolic trans-acetylase and will not replace the A fraction in the acetyl generating system, nor will the A fraction replace the carboxylase in the acetate forming system. Wolfe & O'Kane (90) in studying reaction requirements for  $CO_2$  and acetate exchange in cell-free extracts of *Clostridium butylicum* found a very rapid  $C^{14}O_2$  incorporation. Artificial aging yielded extracts which would rapidly exchange  $C^{14}O_2$  into pyruvate but would not exchange  $CH_3C^{14}OOH$ , and could not conduct the forward decomposition. DPT and Co A were found to be required for the  $C^{14}O_2$  exchange. Furacin stimulated the forward reaction but completely inhibited the incorporation of  $C^{14}$  into pyruvate. The perplexing aspects of formate exchange in *E. coli* are briefly reported upon by Novelli, Gest & Krampitz (91). Removal of Co A from *E. coli* extracts by treatment with ion-exchange resin resulted in the loss of pyruvate utilization and formate exchange activities. Addition of Co A would restore the formate exchange activity but not pyruvate dissimilation. After aging the treated extracts would no longer respond to added Co A, but would exchange after the addition of extracts of *C. butylicum*, *C. pasteurianum*, and *Micrococcus lactilyticus*. Ammonium sulfate fractionation precipitates could be similarly reactivated.

Lieberman (92) has shown that formate has a catalytic effect upon the hydrolytic decomposition of acetyl phosphate by extracts of *C. kluyverii*. Two formyl compounds were found to accumulate. One of the compounds reacted with hydroxylamine and the other with ninhydrin if cysteine was present. Co A was found to be required catalytically for the synthesis of the formhydroxamic acid precursor. The following reaction sequence was postulated:

- $$\begin{aligned}
 & \text{E}_1 \\
 (a) & \text{Acetyl-PO}_4 + \text{Co A} \rightleftharpoons \text{acetyl-Co A} + \text{phosphate} \\
 (b) & \text{acetyl-Co A} + \text{E}_2 \rightleftharpoons \text{acetyl-E}_2 + \text{Co A} \\
 (c) & \text{acetyl-E}_2 + \text{formate} \rightleftharpoons \text{formyl-E}_2 + \text{acetate} \\
 (d) & \text{formyl-E}_2 + \text{acceptor} \rightleftharpoons \text{formyl-acceptor} + \text{E}_2 \\
 (e) & \text{formyl-acceptor} + \text{H}_2\text{O} \rightleftharpoons \text{formate} + \text{E}_2
 \end{aligned}$$

Lactate and glycolate behaved similar to formate thereby indicating lack of enzyme specificity in reaction sequence *C*. Propionate inhibited through competition with formate for the site on  $\text{E}_2$ .

Pine & Barker (93) found that methane-producing bacteria incorporate the deuterium of deuterized water only to the remaining carbon valence of the methyl groups of acetate or methanol in the production of methane from these molecules. Non-randomly distributed deuterium in the methyl groups of acetate was not randomized in the methane produced. It was concluded that the methyl groups were incorporated intactly.

Several publications concerning the dissimilation of citric acid to glyoxylate and succinate have appeared since the original observations by Campbell, Smith & Eagles (94) with *Pseudomonas aeruginosa*. The *P. chrysogenum* system of Olson (95) was reported to decompose citric acid in a similar manner, but unlike the experiments with the *P. aeruginosa* extracts, the data indicated that isocitrate was an intermediate. Saz (96), working with extracts of *P. fluorescens* and *P. aeruginosa*, also found succinate and glyoxylate formation from isocitrate as well as citrate and *cis*-aconitate. The publications of Wheat & Ajl (97) and of Wheat, Wong & Ajl (98) described a Co A independent breakdown of citric acid in *E. coli* by a mechanism not involving isocitric dehydrogenase.

A very curious ATP effect in the anaerobic breakdown of pyruvate by *Clostridium saccharobutyricum* was studied by Weisendanger & Nisman (99). Extracts of the organism would oxidatively decarboxylate pyruvate only in the presence of ADP or ATP. Instead of phosphate esterification occurring, the reaction was accompanied by a liberation of orthophosphate from ATP or ADP. ATP-ase and pyrophosphatase activities were absent, hence the liberation of inorganic phosphate was established as a property of the enzymatic system under study. The authors concluded that the enzyme phosphotransacetylase is the probable activation site. King & Cheldelin (100) found that *Acetobacter suboxydans* possesses a yeast-type carboxylase as is the case with the related pseudomonads. Free acetaldehyde was produced by a purified DPT and  $\text{Mg}^{++}$  dependent enzyme. Other  $\alpha$ -keto acids were attacked to a limited degree.

Strecker & Ochoa (101) have obtained data indicating that the formation of  $\alpha$ -acetolactate by cell-free extracts of *Aerobacter aerogenes* requires two protein components, one of which is present in the protein Fraction A from *A. aerogenes* or *E. coli* (102), and that the oxidative and non-oxidative pathways of pyruvate dissimilation do not share a common intermediate. Alternatively it is suggested that the decarboxylation step might be catalyzed

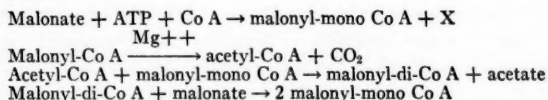
by different proteins, both yielding bound "acetaldehyde." Cell-free extracts of *Proteus morgani* metabolize pyruvate to  $\alpha$ -acetolactate [Kobayashi & Kalnitsky (105)] in a manner similar to that described for *A. aerogenes* by Juni (103, 104).

A study of  $\beta$ -keto acid formation by *C. kluyverii* [Lieberman & Barker (106)] disclosed that the oxidation of *n*-valerate and *n*-caproate in the absence of phosphate resulted in the formation of the respective  $\beta$ -keto acids. Subsequent cleavage was found to yield equimolar quantities of acetyl and propionyl phosphates and the corresponding fatty acids in the case of the  $\beta$ -ketovalerate, and only acetyl phosphate and a fatty acid, presumably butyric, in the case of the  $\beta$ -ketocaproate. The possible reaction mechanisms are discussed.

Tomlinson & Barker (107) and Tomlinson (108, 109) studied  $C^{14}O_2$  and  $C^{14}H_3COOH$  assimilation into the cell amino acids of *C. kluyverii*. With  $C^{14}O_2$  17 amino acids were found to be labeled. The experiments with acetate-1- $C^{14}$  demonstrated that it is a precursor of at least 15 amino acids. The degradation of alanine, serine, glycine, threonine, and aspartic acid revealed that the carboxyl carbons of each of these amino acids was derived mainly from  $CO_2$ . In the case of threonine 42 per cent of the total  $C^{14}$  was found in the  $\gamma$ -carbon atom. With acetate-1- $C^{14}$  the radioactivity found was almost all contained in the  $\alpha$ -carbon atoms. The labeling found in glutamic acid and proline was not in accordance with that predicted by the Krebs' cycle. With  $C^{14}O_2$  90 per cent of the labeling was located in the  $\gamma$ -carboxyl carbon of the glutamic acid and with acetate-1- $C^{14}$  the radioactivity was found equally distributed between the  $\alpha$ -carboxyl carbon and the  $\beta$ -carbon. An attractive explanation is offered in terms of a modified aconitase reaction. The labeling pattern can be explained [Tomlinson (109)] by assuming double bond formation between the central carbon of the *cis*-aconitic acid and the adjacent atom arising from the methyl group of the acetate rather than from the adjacent carbon which originated from the methylene carbon of precursor oxalacetate.

Hayaishi (110) continued his studies on the mechanism of malonate decarboxylation by *Pseudomonas* sp. Completely analogous to the conclusions in the case of succinate decarboxylation [Delwiche, Phares & Carson (111); Phares, Carson & Delwiche (112); Whiteley (113)], it was suggested by him that malonyl-Co A is decarboxylated to acetyl-Co A +  $CO_2$ , malonyl-Co A is regenerated through a transphorase reaction, and as with *Propionibacterium*, acetate can be activated by the ATP-Co A-dependent sequence of reactions. Also in keeping with the succinate decarboxylation studies, it was found that the purified decarboxylase, which required both Co A and ATP for decarboxylation, could be activated by Co A, acetyl-phosphate, and phosphotransacetylase in the absence of added ATP. This latter observation contradicts the findings of Wolfe, Ivler & Rittenberg (114, 115) and Wolfe & Rittenberg (116) who reported that with cell-free preparations of

*P. fluorescens*, acetyl-Co A could not replace ATP although it would stimulate the decarboxylation in the presence of ATP. Considering also their chromatographic evidence for a diactivated malonate they postulate the following reaction system:



It is envisaged that the absolute requirement for small quantities of ATP is a result of the necessity of ATP to activate malonate. Malonyl-di-Co A can replace ATP but acetyl-Co A cannot. The continuing sequence of reactions results in the eventual decarboxylation of the malonyl-mono Co A, which can arise initially only through activation with ATP or malonyl-di-Co A. In view of the replacement of ATP with the transacetylase system as recorded by Hayaishi (110), as well as the findings with the succinate decarboxylase system in *M. lactilyticus* where propionyl-Co A will replace ATP, this postulate of the function of a malonyl-di-Co A must be viewed with caution. Delwiche, Phares & Carson (117) offered evidence that the  $C_1$  product of succinate decarboxylation initially is not  $\text{CO}_2$ . Aged (or otherwise treated) extracts of *Veilonella gazogenes* (*M. lactilyticus*) did not produce  $\text{CO}_2$  from succinate, nor did extracts of *P. pentosaceum*. Nevertheless, when the two extracts were mixed together  $\text{CO}_2$  was produced from succinate. These and other data are used to support the contention that these are at least two different reactions in the decarboxylation process: (a) succinyl-Co A  $\rightarrow$  propionyl-Co A + " $C_1$ " and (b) " $C_1$ "  $\rightarrow$   $\text{CO}_2$ . The nature of the " $C_1$ " is unknown, but it was established that a "malic" enzyme was not responsible for the " $C_1$ "  $\rightarrow$   $\text{CO}_2$  conversion.

#### NITROGEN METABOLISM

The question of hydrogen source for nitrogen fixation was raised by Parker (118). It was considered by him that metabolite hydrogen rather than water was a more reasonable choice of hydrogen donor since the union of nitrogen and hydrogen from this source could yield appreciable amounts of free energy. In his experiments with *Azotobacter* sp. he observed that more nitrogen was fixed under conditions of reduced oxygen tension. He views that nitrogen fixation might represent an alternate form of respiration with  $\text{N}_2$  instead of  $\text{O}_2$  acting as the ultimate hydrogen acceptor. That *Clostridium* can apparently fix more nitrogen per unit substrate under certain conditions (112) is considered as supporting evidence. Pethica, Roberts & Winters (119) contend that no conclusive evidence exists that oxidative initial steps may not occur in the nitrogen fixation process. They found that  $\text{H}_2\text{NOH}$  inhibited both respiration and fermentation in *A. vinelandii*. An interpretation is made in terms of linked enzyme systems with the linkage believed to occur through the mediation of a high energy product of respiration, possibly

$\text{H}_2\text{O}_2$ , which acts as a source of hydroxyl radicals. As concerns the possible role of hyponitrous acid in the fixation reaction, Chaudhary, Wilson & Roberts (120) found that with *A. vinelandii* it inhibited both respiration and fixation. For the fixation process it was found to act as an irreversible non-competitive inhibitor. Hence these investigators believe hyponitrous acid not to be an intermediate. With  $\text{N}_2\text{O}$ , however, significant data for its role as an intermediate is seen in the work of Mozer & Burris (121). Using  $\text{N}_2^{15}\text{O}$  as a tracer they were able to establish incorporation both with *A. vinelandii* and with soybean nodules. Aprison, MaGee & Burris (122) reported more extensively on nitrogen fixation by excised soybean root nodules. Fixation by excised nodules seems established beyond a reasonable doubt.

Nicholas, Nason & McElroy (123) and Nicholas & Nason (124, 125) clarified a role for molybdenum in nitrogen metabolism. Observing a striking decrease in the nitrate reductase activity of cell-free extracts of molybdenum-deficient *N. crassa* and *A. niger*, they identified molybdenum as a trace constituent of nitrate reductase. A detailed study of the purified reductase from *Neurospora* disclosed that the enzymatic transfer of electrons from TPNH to nitrate involved both FAD (or FMN) and molybdenum as electron carriers. Cyanide-poisoned enzyme lost the ability to catalyze the reduction of nitrate to nitrite although it would still reduce flavin with accompanying oxidation of TPNH, thereby establishing the reduction sequence as follows:



Silver & McElroy (126) made growth and enzyme studies on nitrate and nitrite mutants of *Neurospora*. In the cases of the nitrate mutants it was found that the genetic blocks directly or indirectly affected the apoenzyme of nitrate reductase. Of the nitrite mutants studied, one strain was found to have low nitrite reductase and three had low nitrate reductase activity. Two of the mutants accumulated hydroxylamine and nitrite in the culture medium. Pyridoxine was implicated as a result of its requirement in one strain, which under conditions of pyridoxine deficiency displayed nitrite accumulation and diminished nitrite reductase activity. It was suggested that pyridoxine might operate directly in nitrite utilization through union with hydroxylamine to form the oxime. The oxime could be reduced to the amine and free pyridoxal phosphate regenerated through transamination. In the light of their data the authors regard the apparent absence of a particular enzyme as not necessarily fixing the biochemical lesion related to the genetic block. The publication of Lees, Simpson, Jensen & Sørensen (127) stands in further support of hydroxylamine as an intermediate in nitrite metabolism. Strains of *Alcaligenes* sp., *Corynebacterium equi*, and *Nocardia corallina* were found to be capable of forming nitrite from hydroxylamine and from oximes if the latter were added in low concentrations to washed cell suspensions. Krasna & Rittenberg (128), working with cell-free extracts of *P. vulgaris*, obtained nitrate reduction with molecular hydrogen. Klausmeier

& Bard (129) isolated a pyridine nucleotide-requiring ammonium dehydrogenase from *B. subtilis* Marburg. The reverse reaction, the reduction of hydroxylamine to ammonia, was demonstrated.

Much work has been published recently on the metabolism of the individual amino acids, but because of space limitation only a few of the more broadly significant papers shall be cited briefly. Levy & Coon (130) studied the biosynthesis of histidine from radioactive acetate and glucose in an effort to determine the origin of the 5-carbon chain of histidine. With acetate-2-C<sup>14</sup> and unlabeled glucose the histidine of yeast grown in the presence of the radioactive acetate was found to be labeled exclusively in the carbon atom 2 of the imidazole ring. Previous studies by the same workers (131) established formate to be the precursor of the carbon in this position, hence it was concluded that the methyl carbon of acetate can serve as a source of "formate." No radioactivity was found in the 5-carbon chain; therefore acetate or products derived from it are not involved in the biosynthesis of the histidine side chain. With uniformly labeled glucose or with glucose labeled in the carbon 1 position, the isolated histidine was found to be radioactive. Histidine derived from glucose-1-C<sup>14</sup> contained the labeling primarily in position 5. The other carbon atoms contained a small amount of labeling; and in particular carbon atom 2 was found to contain a small although significant level of isotope, thereby establishing that carbon atom number 1 of glucose can also yield "formate." In an investigation of the formation of L-glutamic acid from histidine, Tabor & Mehler (132) isolated a crystalline compound from the urocanic acid degradation by guinea pig liver extract. This compound, which contained bound L-glutamic acid, formic acid, and ammonia, was converted to formyl-L-glutamic acid by aged extracts of histidine-adapted *P. fluorescens*. It therefore appears that formyl-L-glutamic acid is the immediate precursor of L-glutamate in the decomposition of histidine by *P. fluorescens*. Vogel & Bonner (133) have concluded that proline is synthesized in *Neurospora* via glutamic- $\gamma$ -semialdehyde and  $\Delta^1$ -pyrroline-5-carboxylate, with another route existing from exogenous ornithine via the same intermediates. Tamiya (134) claims evidence for the aerobic decomposition of cysteine by *E. coli* via a mechanism substantially different from the anaerobic desulfhydration route. Cysteine decomposition was found to occur only in the presence of oxygen or nitrate, and the liberation of hydrogen sulfide was preceded by deamination. It was proposed that the molecule is first oxidatively deaminated to thiolpyruvic acid, which is decomposed to hydrogen sulfide and "other products" by a reaction mechanism as yet obscure.

Research on the "arginine dihydrolase" system of bacteria has centered about the energetics of the citrulline to ornithine conversion. The rate of conversion by *S. faecalis* extracts is increased by the addition of arsenate [Knivett (135, 136); Slade, Doughty & Slamp (137); and Korzenovsky & Werkman (138)]; and with arsenate present, conversion can occur in the



absence of a phosphate acceptor. Slade, Doughty & Slamp (137) isolated a pseudomonad from soil which utilized citrulline as the sole source of energy for growth. Cell-free extracts required ADP (or AMP), inorganic phosphate, and divalent ions for the citrulline to ornithine conversion. No myokinase was present in the extracts, hence it was judged that AMP as well as ADP could act as a phosphate acceptor. The action on citrulline appeared to be a phosphorolysis. Oginsky & Gehrig (139) visualized this possibility in their earlier report on the *S. faecalis* system, and Knivett (136) suggested phosphocitrulline. No workers have succeeded in isolating a phosphorylated intermediate, however.

Cell-free culture filtrates of *B. subtilis* obtained by Thorne *et al.* (140) were found to contain glutamyl polypeptides, with the D-glutamic acid content varying from 20 to 80 per cent. Growth in static culture usually produced a peptide higher in glutamate than that produced under conditions of agitation. Williams & Thorn (141, 142), in a series of excellent experiments, isolated an exoenzyme preparation from *B. subtilis* culture filtrates which would catalyze a transamidation reaction in which the  $\gamma$ -glutamyl radical of glutamine is transferred to D-glutamic acid,  $\alpha$ -D-glutamyl-D-glutamic acid, and probably to DL-aspartic acid and glutamine itself to form glutamyl peptides. Both D- and L-glutamine were utilized for the synthesis of glutamyl peptides. It was observed that with  $\gamma$ -D-glutamyl-D-glutamate the enzyme preparation would catalyze a trans-peptidation resulting in the formation of free glutamic acid and elongation of the peptide chain. These significant results demonstrate peptide chain elongation without hydrolysis and net loss of peptide bonds.

A role for aspartic acid in purine biosynthesis was established by the research of Wahba & Shive (143) and Wahba, Ravel & Shive (144). Gots & Love (145) obtained additional evidence in support of the role of 4-amino-5-imidazole carboxamide in purine synthesis. Friedman & Fox (146) found that the accumulation of this compound by purine and *para*-amino benzoic acid auxotrophs of *E. coli* was controlled by the potassium concentration. Aspartic acid may also be involved in the synthesis of pyrimidines (147, 148, 149) as is ureidosuccinate (148, 149, 150).

The most significant advances in the fields of nucleic acid function and protein synthesis are embodied in the work of Gale & Folkes (151), who were successful in the demonstration of amino acid incorporation and exchange, and over-all protein synthesis with suspensions of supersonically disintegrated staphylococci (*Micrococcus pyogenes* var. *aureus*). After DNA and RNA extraction with molar sodium chloride, they effectively accomplished the exchange of  $C^{14}$ -labeled glutamic acid, and other amino acids. In the presence of a complete mixture of amino acids, glutamate incorporation was accompanied by an increase in the protein-nitrogen content of the preparation. The synthesis of catalase,  $\beta$ -galactosidase, and the enzymes of the glycolytic system was also demonstrated. The purine or pyrimidine

components or the DNA or RNA as such were found necessary for the synthetic process. The net incorporation of  $C^{14}$ -labeled uracil but not labeled thymine lead the authors to conjecture that the preparation was synthesizing ribonucleic acid as well as protein. The importance of this superlative effort lies in the direct demonstration of a nucleic acid function in protein synthesis, and the final accomplishment of cell-free protein synthesis.

#### VITAMINS, COENZYMES AND INORGANIC IONS

Plaut (152) has undertaken a study of the biosynthesis of riboflavin. Using *Ashbya gossypii* as the experimental organism, he traced the incorporation of  $C^{14}$ -formate,  $C^{14}$ -bicarbonate,  $CH_3C^{14}OOH$ ,  $C^{14}H_3COOH$ ,  $NH_2C^{14}H_2COOH$ , and  $NH_2CH_2C^{14}OOH$ .  $C^{14}$ -formate appeared in carbon 2, but with  $C^{14}$ -bicarbonate and with  $CH_3C^{14}OOH$  the labeling appeared in carbon 4. With  $C^{14}H_3COOH$  or totally-labeled glucose, carbon 4 remained unlabeled. With the labeled glycine, essentially all of the radioactivity was in the C-4a and C-9a portion of the molecule. With regard to the aromatic ring portion of the riboflavin molecule, Plaut (153) considered the possibility that 2-carbon fragments may be the basic units in its biosynthesis. Consistent with the data of Plaut (152), McNutt (154) demonstrated an increase in riboflavin yield with *Eremothecium ashbyii* through the furnishing of free purines. Isotopic adenine, when added to the growth medium, was incorporated into riboflavin in the 6,7-dimethyl isalloxazine portion of the molecule. The ureido carbon of the adenine remained essentially unincorporated.

Menadione may have a general importance in certain electron transport systems (155, 156, 157). Wosilait & Nason (155) isolated a pyridine nucleotide linked menadione reductase from *E. coli*, but were unable to establish a flavin linkage using an acid resolution procedure. Dolin (156), working with *S. faecalis*, isolated a DPNH-menadione reductase which would show little activity in the absence of FAD or FMN. Flavin resolution was accomplished by growth in a flavin-deficient medium. Cormier & Totter (157) with *Achromobacter fischeri* isolated an FMN-dependent menadione reductase which would couple with cytochrome-*c*. They also observed both cytochrome-*c* and menadione inhibition of cell free bacterial luminescence, and thereby suggest that with this hydrogen transfer system from flavin through menadione to cytochrome-*c*, the inhibition of luminescence is a result of competition with the luminescent pathway for the hydrogens of the reduced flavoprotein. Both Dolin (156) and Cormier & Totter (157) recognize the possible relation to the Slater factor (158).

Purko, Nelson & Wood (159) studied pantoate synthesis in a strain of *Bacterium linens* that required either PA or PABA for growth. Pantoate and ketopantoate substituted for PABA and were equivalent to PA in growth response.  $\alpha$ -Ketoisovalerate enhanced growth when PABA concentration was limiting. Sulfanilamide and salicylate would inhibit growth on PABA,

but PA, pantoate and ketopantoate would noncompetitively reverse the inhibition.  $\alpha$ -ketoisovalerate and pantonine were found to be weak competitive antagonists. The interpretation given is that PABA is required for the conversion of  $\alpha$ -ketoisovalerate to ketopantoate and, of the possible pathways of pantoate synthesis as depicted in Fig. 3, the route postulated by Kuhn & Wieland (160), involving  $\alpha$ -ketoisovalerate and ketopantoate, is favored by their evidence with *B. linens*. In their most recent report, Purko, Nelson & Wood (161) announced the isolation of cell extracts from an *E. coli* mutant which would conduct the hydroxymethylation of  $\alpha$ -ketoisovalerate

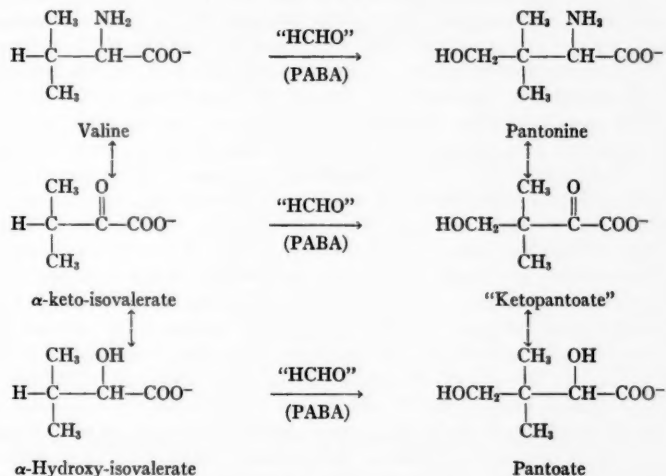


FIG. 3. Pathways of pantoate synthesis [Purko, Nelson & Wood (159)].

with formaldehyde to form ketopantoate. Dialyzed, Dowex-1-treated, or ammonium sulfate-precipitated extracts were inactive unless supplemented with yeast extract or a mixture of tetrahydrofolic acid, metallic ions, glutathione, and either methionine or tryptophane. Thus, tetrahydrofolic acid is strongly implicated in the 1-carbon transfer process approximately ascribed to PABA in the intact *B. linens* cell. It is significant to note that tetrahydrofolic acid has been demonstrated as a cofactor in the glycine-serine interconversion in pigeon liver preparations [Blakley (162)]. Likewise, in the case of cells of *S. faecalis* R, serine synthesis was seen to require a form of folic acid similar to but not identical with leucovorin [Lascelles & Woods (163)].

Mandles, Koppleman & Hanke (164) carried out the enzymatic decarboxylation of lysine, tyrosine, and glutamate with bacterial enzyme prep-

arations in the presence of 99 per cent  $D_2O$ . The finding of not more than one deuterium atom being introduced on the amino carbon that is formed is convincing evidence in support of the Westheimer mechanism (165), which holds that the hydrogen atom on the amino carbon remains fixed throughout the reaction process. Only one deuterium atom would be introduced. This observation excludes the hypothesis of Werle & Koch (166) which proposes the removal of the amino hydrogen prior to decarboxylation, and hence the overall introduction of two deuterium molecules from the solvent.

Gooder & Happold (167) studied the effect of structural analogs of tryptophan on the tryptophanase reaction in *E. coli*. They suggested that the apoenzyme and coenzyme are linked through the coenzyme phosphate group, the coenzyme and substrate are joined through the aldehyde group of the coenzyme and the amino group of the substrate, and that the substrate may be attached to the coenzyme through the ring nitrogen atom and the terminal carboxyl group. Histidine decarboxylase, heretofore questionable with regard to its similarity to other amino acid decarboxylases, has been shown also to have a pyridoxal-phosphate and metal requirement. Guirard & Snell (168) accomplished resolution of a histidine decarboxylase from the *Lactobacillus* 30a of Rodwell (169) by growth of the organism in a defined medium containing *D*-alanine and a minimal level of pyridoxine. Ferric or aluminum ions were required for enzyme activation. Saz & Brownell (170) have reported that the *D*- and *L*-cysteine desulfhydrases isolated from aureomycin-resistant and aureomycin-sensitive strains of *E. coli* are not enhanced in their activity by the addition of pyridoxal phosphate. In the reviewer's own laboratory, however, [Metaxas & Delwiche (171)] an *L*-cysteine desulfhydrase from *E. coli* 58 was over 90 per cent resolved for pyridoxal phosphate by a simple alumina grinding technique. Marr & Wilson (172) isolated an alanine racemase from *Brucella abortus* which racemized alanine without exchange occurring between the amino group and ammonium ions. The hypothesis of Leloir (173), which visualizes the rearrangement of a Schiff's base intermediate, is supported by this observation.

#### OTHER STUDIES

Weiss, Gilvarg, Mingioli & Davis (174) announced the isolation of a compound, designated as "prephenic acid," which is considered by them to be an immediate precursor of an aromatic biosynthetic intermediate. Prephenic acid (Fig. 4) is excreted by certain phenylalanine requiring mutants of *E. coli*. Under conditions of mild acidity it is converted to phenylpyruvic acid. Extracts of wild type *E. coli*, but not of the phenylalanine requiring mutants, can also aromatize the molecule with the production of phenylpyruvic acid. Decarboxylation and aromatization are presumed to result from proton attack on the ring hydroxyl group. This is the first occasion of an actual enzymatic aromatization step being placed under experimental observation. Mitsuhashi & Davis (175, 176) isolated enzyme

systems from mutants of *A. aerogenes* and *E. coli* which would convert quinic acid to 5-dehydroquinic acid and 5-dehydroquinic acid to 5-dehydroshikimic acid. In the former case the enzyme was found to be DPN specific. Limited distribution of the quinic dehydrogenase lead the authors to conclude that quinic acid probably is not an intermediate in aromatic biosynthesis. Tatum, Gross, Ehrensward & Garnjobst (177) concluded that the synthesis of aromatic rings in *Neurospora* follows a pathway similar to that in *E. coli*. Dehydroshikimic acid, the normal precursor of shikimic acid, was accumulated by the mutant they studied. The accumulated dehydroshikimic acid was apparently converted to protocatechuic acid.

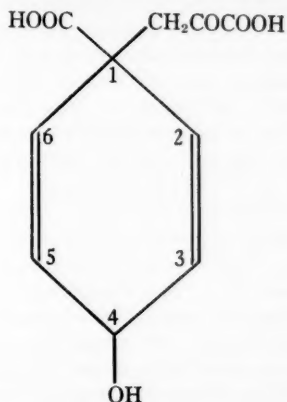


FIG. 4. Prephenic acid [after Weiss *et al.* (174)].

In rebuttal to the criticisms of Baalsrud & Baalsrud (178), Umbreit (179) has announced that a reinvestigation of the sulfur oxidation system of *Thiobacillus thiooxidans*, using radioactive phosphorus, has revealed that labile phosphate ( $\Delta_7$ ) is formed during sulfur oxidation and that this phosphate is released later when  $\text{CO}_2$  is supplied and fixation occurs. Baalsrud & Baalsrud (178) had reported that they were unable to find significant phosphate exchange related to sulfur oxidation and carbon dioxide fixation, and that they found no ability to fix carbon dioxide except during the simultaneous oxidation of sulfate. Newburgh (180) has recorded that in his laboratory *T. thiooxidans* fixed measurable amounts of  $\text{C}^{14}\text{O}_2$  in the absence of simultaneous sulfur oxidation. On the other hand he was able to demonstrate only slightly greater  $\text{C}^{14}\text{O}_2$  fixation with cells previously kept aerobic than with these kept anaerobic; and, during the delayed fixation of carbon dioxide, no release of inorganic phosphate could be demonstrated. Newburgh (180) recognizes that his data on phosphorus levels in the medium may not adequately reflect internal changes.

Pine & Barker (181) and Pine, Haas & Barker (182) have continued their studies on the metabolism of *Butyribacterium rettgeri*. By means of tracer experiments they have provided evidence that the characteristic incorporation of large amounts of carbon dioxide into doubly-labeled acetate does not involve a reverse di- or tricarboxylic acid cycle, the glycine-serine interconversion, or any other reaction sequence which would involve methyl-labeled pyruvate as an intermediate. With  $C^{14}$ -labeled lactate the data showed that the carbon dioxide originates mainly from the lactate carboxyl carbon and the acetate from the 2 and 3 positions.  $C^{14}$ -formaldehyde is incorporated into every carbon atom of the fermentation products of glucose, and with such a pattern of labeling as to indicate the formation of formaldehyde from carbon dioxide in the case of carbon dioxide assimilation. The fermentation of glucose to DL-lactate, acetate, butyrate, carbon dioxide, and hydrogen was found to be dependent upon the iron supply in the medium. Iron-deficient cells conducted a predominantly lactic fermentation when compared to the iron-sufficient cells, although the exchange of carbon between lactate and its oxidation products, acetate and carbon dioxide, was found to be accelerated under conditions of iron deficiency. The lactate and acetate derived from the fermentation of glucose-1- $C^{14}$  were found to be labeled mainly in the methyl group with about 20 per cent of the  $C^{14}$  appearing in the other carbon atoms. Thus it is suggested that the glycolytic sequence provides the main pathway for glucose degradation.

Hofmann & Panos (183), Hofmann, Panos & Tausig (184), and Hofmann, Jucker, Miller, Young & Tausig (185) studied the biotin replacing ability of long chain cyclopropane fatty acids. In addition to having the ability to substitute for biotin in the nutrition of *Lactobacillus arabinosus*, *L. casei*, and *L. delbrueckii*, two members of the series, lactobacillic acid and dihydrosterculic acid, stimulated the growth of a strain of *Lactobacillus acidophilus* which was not dependent upon an external source of biotin. *Clostridium butyricum*, although stimulated by oleic acid and *cis*-vaccenic acid, was found to be inactive toward the octadecanoic acids of the cyclopropane series. It is concluded that the growth-promoting activity of this series is dependent upon a chain length of more than 11 carbons. *Trans*-DL-2,3-methylenonanoic acid and *trans*-DL-2,3-methyleneundecanoic acid failed to support the growth of the test microorganisms and in some cases were growth inhibitory.

Chambers & Delwiche (26) concluded that the previously reported biotin effect in succinate decarboxylation (186) must be an indirect response. Since biotin was apparently not involved in the biosynthesis of pantothenic acid, Co A, and the decarboxylation intermediate, succinyl-Co A, it was conjectured that the demonstrable function in the biotin-deficient intact cell may be a result of an involvement in the " $C_1$ " metabolism of the decarboxylation process, or in some indirect function in the protein biosynthesis of the cell.

Lieberman & Barker (187) demonstrated the production of  $H_2O_2$  by a dried cell preparation of the obligate anaerobe *C. kluyverii* during the oxidation of butyrate. Partial inhibition of the butyrate oxidation occurred. The accumulation of a  $\beta$ -ketoacid, presumably acetoacetate, suggested inhibition by interference with the fission of acetoacetyl-Co A.

Dohner & Cardon (188) reported on an interesting ecological relationship between two strains of *E. coli* isolated from the rumen. Together, but not separately, they would ferment lysine with the production of one molecule each of acetate and butyrate and two molecules of ammonia. Essentially the reaction appears to be "Stickland" oxidation-reduction requiring only one molecular type, and involving the hitherto unreported direct cleavage of the carbon chain of the amino acid.



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## ANTIBIOTICS<sup>1</sup>

BY HARRY EAGLE AND ARTHUR K. SAZ

*Section on Experimental Therapeutics, Laboratory of Infectious Diseases,  
National Microbiological Institute, National Institutes of  
Health,<sup>2</sup> Bethesda, Maryland*

The search for new antibiotics continues, with no indication that the pace has been tempered by the relatively unrewarding harvest in 1953 and 1954. Each year sees the discovery of hundreds of new agents, ranging from amphotericin through hygromycin and pyridomycin to vinaceticin. The published material probably comprises only a fraction of the active products discovered; but almost all prove to be of little or no therapeutic significance by virtue either of their toxicity, instability, or ineffectiveness *in vivo*. As microbial products which have an antimicrobial effect, they would still be of interest if their mode of action, physiological function, or biosynthetic pathways were understood. In the absence of such information, or of experimental approaches to it, and in the absence of a practical application, the fact that a given organism produces a product which is toxic for certain other organisms becomes a relatively sterile datum. No antibiotic has recently been found comparable in practical importance to penicillin, streptomycin, the tetracyclines, or chloramphenicol. At least for the time being erythromycin is finding an important application in the treatment of staphylococcal infections resistant to other antibiotics. The importance of anisomycin in the treatment of protozoal infections (1, 2, 2a) and of fumagillin in amebiasis remains to be assessed. The polypeptide antibiotics (tyrothricin, bacitracin, subtilin, polymyxin, and their congeners) have limited usefulness in local application, or in careful parenteral use as last-resort measures in infections resistant to other forms of therapy.

A number of reports deal with the activity of various antibiotics in the treatment of experimental virus infections (3 to 8) and experimental cancer (9 to 14). The important implications of these data are self-evident; but it must be emphasized that the effects so far described are incomplete, and that the information now available does not justify routine clinical use in these directions.

Growing interest is evident in the screening of antibiotics for efficacy in the treatment of fungal (15, 16) and protozoal (17, 18, 19) infections. It is

<sup>1</sup> No attempt has been made in the present review to cover in its entirety the vast literature on the antibiotics for the years 1953-1954. Instead, a limited number of topics have been arbitrarily selected for discussion; and with these, the references cited are representative rather than complete. In order to clarify their relation to previous work, it has been found necessary to include a number of papers which antedate 1953.

<sup>2</sup> Public Health Service, U. S. Department of Health, Education, and Welfare.

too soon to assess the clinical value of the agents which have been tested and found active.

### BIOSYNTHESIS AND CHEMISTRY

For obvious reasons, much of the information concerning the optimal conditions for the production of the major antibiotics, and of the biosynthetic mechanisms involved in their formation, is closely held by the manufacturers. Nevertheless, some data along these lines are available.

#### PENICILLIN

All of the 11 amino acids detected in corn steep liquors were found to be taken up by *Penicillium chrysogenum* prior to the period of rapid penicillin formation; and all, plus glutamine, were found in the mycelium. When cultures were grown in media containing inorganic N as the sole nitrogen source, the same 12 amino acids were found in the mycelium (20). With  $S^{35}$ -labelled material, Stevens *et al.* have found that L-cyst(e)ine, methionine, and glutathione were utilized in preference to sulfate by *P. chrysogenum* in the production of penicillin (21). DL-Homocysteine, L-cystic acid, and taurine were also utilized, but not as effectively as L-cystine (22). The authors leave open the question as to whether one or more of these compounds are normal intermediates in the biosynthesis of the antibiotic. However, Arnstein & Grant (23) suggest that cystine is in fact a direct precursor since, with labelled amino acid, the ratios of  $C^{14}$ ,  $N^{15}$ , and  $S^{35}$  were the same in both mycelial cystine and penicillin as in the cystine added to the medium. A method of producing  $S^{35}$ -labelled penicillin has been described by Perret (24). The carboxyl group of valine was incorporated as the carboxyl group of penicillin (24a).

$C^{14}$ -carboxyl-labelled phenylacetic acid is converted to penicillin with the same specific activity as the acid, 82 per cent of which is associated with the phenylacetic acid residue, 1.5 per cent with the  $\beta$ -lactam ring, 0.2 per cent with glycine residue, and 5 per cent with the acetic grouping (25). No less than 58 per cent of the precursor was recovered in the antibiotic, and the protein and lipid of the mycelium were also labelled (26).

The continuous feeding of glucose (0.03 per cent per hour) led to a significant increase in the production of penicillin (27). Penicillin formation was exceedingly sensitive to KCN, a concentration of  $2 \times 10^{-6}$  M causing 75 per cent inhibition; and 2,4-dinitrophenol inhibited penicillin formation at concentrations which had little effect on respiration (27a).

A new penicillin V, with a phenoxymethyl side group, has been reported to be relatively acid stable and therefore particularly suited for peroral treatment in man (28). Two more relatively insoluble salts of penicillin G have been prepared. "Benethamine" penicillin (N-benzyl- $\beta$ -phenylethyl amine salt of penicillin G) has a solubility of 0.1 per cent in water, and has been recommended for use in aqueous suspension (29). It is apparently intermediate between procain penicillin and "Bicillin" with respect to solubility,



rate of absorption, and the height and duration of the blood levels afforded. Hydrabamine penicillin G [N,N'-bio-(dehydroabietyl)-ethylenediamine-dibenzyl penicillin] has a solubility of 0.005 to 0.01 per cent (29a).

Cephalosporin N, one of six antibiotics known to be produced by *Cephalosporium*, has been identified as a new type of penicillin which is penicillinase sensitive, much more hydrophilic than penicillin G, as active as penicillin G against gram negative bacteria, but only one hundredth as active against gram positive bacteria (30). It was subsequently shown to contain penicillaminic and d- $\alpha$ -amino adipic acids (31), and finally identified by Newton & Abraham as (D-4-amino-4-carboxy-n-butyl) penicillin (32, 32a). Similar hydrophilic penicillins with a comparably low ratio of activity against gram positive and gram negative bacteria have been identified in small amounts in filtrates of *P. chrysogenum* (33).

#### STREPTOMYCIN

In a synthetic medium,  $\text{CoCl}_2$  at 0.002 to 0.009 *M* enhanced the production of streptomycin by *Streptomyces griseus* (34). By the addition of uniformly labelled glucose to the medium, radioactive streptomycin was produced with a specific activity of 0.01 to 0.025  $\mu\text{c. per mg.}$  One per cent of the glucose was incorporated (35).

The guanidine group of streptomycin has been shown by tracer studies to derive largely, if not entirely, from  $\text{CO}_2$ , with arginine as a possible intermediate. A number of compounds containing a guanidine grouping, or suitable precursors of it, were shown to be converted by *S. griseus* into an unidentified compound containing guanidine groups which may be an intermediate in the biosynthesis of streptomycin (36).

#### CHLORAMPHENICOL

The addition of phenylalanine to the growth medium has been found to increase markedly the amount of chloramphenicol formed (37, 38).  $\alpha$ -Aminobutyrate, norvaline, leucine, tyrosine, methionine, lysine, and tryptophan were less effective, but also active. Chloramphenicol was produced in good amounts by *Streptomyces venezuelae* in a synthetic medium with glucose, maltose, starch and glycerol, but not sucrose, as a carbon source.

Gottlieb, *et al.* (39) also found that phenylalanine stimulated chloramphenicol production during the active growth phase of *S. venezuelae*, and by as much as 500 per cent; p-nitrophenylserinol caused a 280 per cent increase. A number of amino acids, in particular leucine, isoleucine, methionine, and tryptophan, were also effective; but norleucine was the most active precursor of all the compounds studied, despite the fact that it is apparently unrelated structurally to chloramphenicol. On both synthetic and non-synthetic media, the maximum production of chloramphenicol followed the maximum amount of growth of *S. venezuelae* (40). In nonsynthetic medium (Tryptone-glycerine) there was first a drop in pH and then a rise. At this point, the mycelium lysed, and there was an increase in the chloramphenicol

and  $\text{NH}_4^+$  content of the medium. However, there was no indication that the antibiotic had been stored in the cells. The addition to the medium of high concentrations of chloramphenicol, equal to the amount normally formed, inhibited the production of the antibiotic; and on the removal of the antibiotic from the medium, synthesis resumed. Ehrlich *et al.* (41) were unable to show that *S. venezuelae* produced chloramphenicol in nature. If the antibiotic was added to soil, it could be identified as such for at least six weeks. Further, chloramphenicol was produced in sterile soil inoculated with the organism. However, no detectable amount of antibiotic was produced in inoculated natural soil; and in fields in which *S. venezuelae* was a part of the normal flora, no antibiotic could be detected.

#### THE TETRACYCLINES

The structures of the three tetracyclines have now been definitely established (42 to 45); and tetracycline, the parent compound, has been prepared by reductive dechlorination of chlortetracycline (46, 47).

Several groups of workers have reported the separation of the tetracyclines by chromatography and other means (48, 49, 50). In chromatograms sprayed with p-dimethylaminobenzaldehyde, chlortetracycline formed a dirty yellow color, and oxytetracycline became blue-green (48). Cooper (51) has listed simple chemical reactions to distinguish the various antibiotics.

Sucrose has been found to be the best carbon source for the biosynthesis of chlortetracycline, and a peanut oil meal with a low fat content proved to be the best medium (52). The yield of chlortetracycline in this medium was 1300  $\mu\text{g}$ . per ml. The same authors describe methods for the extraction of the antibiotic.

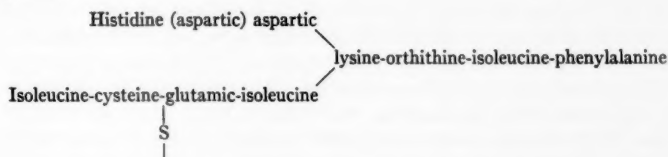
Biffi *et al.* (53) observed that in cultures of *Streptomyces aureofaciens*, chlortetracycline production began only after a certain amount of mycelium had been formed, and continued during the late, lower growth rate of the organism. When the medium was depleted of sucrose, antibiotic production ceased.  $\text{K}_2\text{HPO}_4$  and  $(\text{NH}_4)_2\text{SO}_4$  at concentrations in excess of 0.3 and 0.6 per cent, respectively, decreased the amount of antibiotic formed. The addition of 0.4 per cent  $(\text{NH}_4)_2\text{SO}_4$  lowered the rate of formation of mycelium for the first 24-hr. period, but, after 48 hours, the amount of mycelium exceeded the controls, and there was then a parallel increase in antibiotic production.

#### OTHER ANTIBIOTICS

The molecular weight of polymyxin has been estimated to be 1150 (54). On hydrolysis, it yielded 1 molecule each of isopelargonic acid, leucine, and phenylalanine, 6 molecules of  $\alpha$ - $\gamma$ -diaminobutyric acid, and 2 molecules of threonine.

The extensive studies of Craig, Hausmann & Weisiger (1952 to 1954) (55), and of Newton and Abraham and their co-workers (56, 56a), on the amino acid composition of bacitracin A have led to its description as a polypeptide with a molecular weight of approximately 1470. This peptide yields

1 molecule each of D-ornithine, L-lysine, L-histidine, D-glutamic acid, L-cysteine, D-phenylalanine, L-leucine, and 2 molecules each of DL-aspartic acid and L-isoleucine on acid hydrolysis. More recently Lockhart & Abraham (57) have demonstrated by partial hydrolysis and electrophoretic fractionation the presence of a number of peptides consistent with the amino acid sequence:



There was an indication that the histidine and phenylalanine residues were linked to form a ring. The valine identified in hydrolysates of the commercial product probably derived from bacitracin B. With the latter, the peptides isolated suggested the amino acid sequence lysine-ornithine-valine-phenylalanine.

The varying nephrotoxicity of commercial bacitracin has been shown by Codington (57a) to be due to the varying proportions of bacitracins A and F. Both are nephrotoxic, but only the former is bactericidal. Evidence suggests that the conversion of bacitracin A to F involves the transformation of a thiazoline to a thiazole ring.

Flynn *et al.* (58) have reported initial studies on the chemical properties and degradation of erythromycin with a view to determining its structural formula. Chromatographic methods for the separation of erythromycin and magnamycin, as well as other basic butyl alcohol-soluble antibiotics, are described by Sokolski *et al.* (59).

A new actinomycin (D) has been crystallized from cultures of *Streptomyces parvullus*, and on acid hydrolysis yielded threonine, sarcosine, proline, valine, and N-methyl valine (60). Brockmann & Franck (61) have crystallized desaminoactinomycin C. Actinomycins A, B, C and D have been separated chromatographically (62). The chemical identification of puromycin (63) has been followed by its total synthesis (64). Acker (65) has shown that with *S. griseus* 3570, the best nitrogen sources for the production of candidin were L-asparagine and L-histidine, that the best carbon sources were glucose and mannose, and that K, Mg, Fe, Zn, PO<sub>4</sub> and SO<sub>4</sub>, but not Mn, were essential for growth and the production of the antibiotic. Cooper (51) has described a number of simple color reactions designed to differentiate among penicillin, streptomycin, chloramphenicol, erythromycin, chlortetracycline, and oxytetracycline. Similar presumptive tests have been described by Fischbach & Levine (66).

In an interesting and significant paper, Erlanger & Goode (67) have found that a synthetic acyclic decapeptide, previously prepared in the same laboratory (68) with the same amino acid composition and sequence as

gramicidin S, was bactericidal for *Escherichia coli* and *Micrococcus pyogenes*, and that its activity against these two organisms was, respectively, approximately 1/12 and 1/40 that of gramicidin. The surviving bacteria were found to possess enzymes capable of destroying or neutralizing the compound. Contrary to earlier reports, therefore, the cyclic structure of gramicidin is apparently not essential for antibacterial activity.

Countercurrent distribution permitted the separation of neomycin A and catenulin from neomycins B, and C, and framycetin, but did not permit differentiation within these groups (67a).

A novel method for the concentration and purification of bacteriolytic antibiotics has been described, in which the material is passed through a chromatographic column consisting of the heated bacteria, admixed with an inert carrier (69).

### MODE OF ACTION

*General.*—The mechanisms whereby the antibiotics effect their cytotoxic action remain obscure. Whether with penicillin, streptomycin, or the so-called broad-spectrum antibiotics, the large number of observations describing effects on metabolic activities cannot yet be incorporated into a coherent and meaningful pattern. The difficulty of distinguishing between a biochemical change which is a direct effect of the antibiotic, and a secondary effect in a damaged cell continues to complicate the interpretation of the data. An interesting development has been the recognition that a number of antibiotics other than penicillin are maximally effective in an environment favorable to growth and multiplication (70, 71, 222) (cf. page 185 in re streptomycin). The enhancement of penicillin activity by cobalt (72) has been found to apply to streptomycin, bacitracin, and penicillin-streptomycin combinations, but not to chloramphenicol, chlortetracycline, oxytetracycline, or polymyxin (73, 74). Similarly, cobalt has been found to potentiate the action of tyrothricin and bacitracin in combination (75). The action of a number of antibiotics has been found to be reversed by normal metabolites (*vide infra*); and Pittillo & Foster (76) have made the provocative observation that in a number of such cases, compounds which inhibit the synthesis of the reversing metabolite potentiate the action of the antibiotic.

Olitzki (76a) has described a general metabolic effect of antibiotics which is, however, difficult to relate to specific modes of action. Non-multiplying bacteria exposed to penicillin, streptomycin, chlortetracycline, oxytetracycline or chloramphenicol for 24 hr. at 37°C. remained viable, but had lost their ability to form H<sub>2</sub>S from appropriate precursors. With *Proteus vulgaris* the addition of bacterial extracts or of pyridoxal phosphate reactivated the system, as did the addition of penicillinase to penicillin-treated bacteria. With all the strains, reactivation occurred in subculture in antibiotic-free media.

That host defenses, apart from antigen-antibody reactions, supplement the action of antibiotics in bringing about cure seems clear; but the precise

mechanisms remain to be clarified, as does the question whether the drug and the host act independently, or whether exposure to the drug thereby renders the bacteria abnormally susceptible to cellular or humoral defenses. A number of workers have found that the treatment of bacteria with penicillin or streptomycin sensitizes the organisms to phagocytosis (77 to 79), and, in some experiments, even when the antibiotics were used in sub-bacteriostatic levels (80). The fact that chlortetracycline, oxytetracycline, and chloramphenicol are therapeutically effective at concentrations which *in vitro* are growth inhibitory rather than actively bactericidal, clearly suggests the active participation of as yet unspecified host defense mechanisms. The continuing death of group B streptococci in an experimental mouse infection long after the penicillin had fallen to ineffective levels (81) and the decreased virulence of penicillin-treated but viable type III pneumococci (81, 82) further indicate the increased vulnerability of some bacteria to the host after their exposure to the antibiotics. With a number of other organisms, however, including type I pneumococci and group A streptococci, there was no indication that host defenses continued to operate at an enhanced level after the antibiotic had fallen to ineffective levels. In such cases, in the absence of specific antibody (83), the bactericidal action *in vivo* stopped abruptly, and the surviving organisms recovered to cause a fatal infection (81, 84). Consistent with the latter findings, Hemmer (85) could demonstrate no effect by tetracyclines, chloramphenicol, or polymyxin B on phagocytosis *in vitro*; and, in experimental type pneumococcal infections in rabbits, Gowans (85a) found no evidence that leucocytes assisted penicillin or oxytetracycline in the elimination of the organisms. Lesions were sterilized as rapidly in animals rendered leucopenic with benzene as in normal controls. Specific antibody was found to supplement the antibacterial activity of chloramphenicol on *Salmonella typhosa*, but the drug apparently functioned additively and independently (86).

#### PENICILLIN

The mechanism whereby penicillin exerts its cytotoxic effect remains obscure. The suggestion that it impairs the ability of the cell to assimilate glutamic acid, and that the development of resistance is associated with a decreased requirement for this amino acid (87 to 89) apparently does not explain its general mode of action. Strains of *Bacillus subtilis* (90) and of *M. pyogenes* (91) which did not require glutamic acid were nevertheless normally sensitive to penicillin.

*Protein and nucleic acid metabolism.*—More recently, Gale & Folkes (92) have presented evidence that the incorporation of labelled glutamic acid by washed suspensions of *M. pyogenes* may involve an exchange between the free amino acid and glutamic acid residues in the cell protein. This process was found particularly sensitive to inhibition by penicillin and bacitracin, although other antibiotics were also inhibitory, and the incorporation of other acids was also inhibited. This marked inhibition of glutamic acid in-

corporation by penicillin and bacitracin was not observed when the organisms were in a medium containing a full complement of amino acids and in which there was presumably active protein synthesis. The reverse relationship was observed with chloramphenicol, chlortetracycline, and oxytetracycline which, in growth-inhibitory concentrations, almost completely inhibited the incorporation of glutamic acid from a complete medium, but caused only a slight inhibition of its incorporation by washed bacteria from an incomplete medium (92). The incorporation of phenylalanine was inhibited by the latter antibiotics to an even greater extent than that of glutamic acid (93).

Hotchkiss' finding (94) that penicillin-treated *M. pyogenes* released polypeptides into the medium in amounts equivalent to the amino acid nitrogen utilized, suggests a derangement of protein synthesis. Simmonds & Fruton (95) found that penicillin inhibited both the assimilation of glycine and the growth response to glycine in a gram-negative bacillus. The latter inhibitory effect was reversed by the addition of L-leucyl glycine. They suggested that penicillin in this instance inhibits peptide synthesis (96). More recently, Carr & Macheboeuf (97) have found that penicillin at concentrations of 10 to 50 units per ml. caused only partial growth inhibition in *Clostridium sporogenes* but markedly inhibited its peptidase activity.

A number of workers have suggested that penicillin causes a derangement of nucleic acid synthesis. Thus, Mitchell & Moyle (98 to 100) found that in *M. pyogenes* penicillin caused an increase in the free nucleotide and nucleoside content of the cell, less free purine, a decreased synthesis of an unidentified cell component, and decreased RNA (ribonucleic acid) and DNA (deoxyribonucleic acid) synthesis. Park (101, 102) demonstrated that penicillin-treated staphylococci accumulated three distinct uridine-5'-pyrophosphates, all containing an amino sugar linked to a phosphoric acid group by an acetal-like bond. One of these compounds contained an L-alanine residue, and one a peptide composed of L-lysine, D-glutamic acid, and three alanine residues. Strominger (103) has found that minute amounts of these compounds are also present in normal staphylococci, but that their concentration is increased at least 5- to 10-fold under the influence of penicillin. Penicillin inhibited the normal incorporation of uracil-2-C<sup>14</sup> into uracil and cytosine of nucleic acid; but it was incorporated into the accumulated abnormal nucleotides. Strominger has described at least two such compounds in addition to the three described by Park: an acetylhexosamine and a cytosine-5'-phosphate. Gros-Doulcet & Gros (104) have found that in *M. pyogenes*, penicillin at 0.7 unit per ml. inhibited the synthesis of guanosine-oxidizing enzyme, and before either the oxidative capacity for glucose or the synthesis of protein and nucleic acid had been affected. Macheboeuf & Gros (105) have recently summarized their extensive studies pointing to a derangement of ribonucleic acid metabolism as the primary point of attack of penicillin.

Gale & Folkes (106) have recently made the important observation that cell particulates prepared from *M. pyogenes* by sonic oscillation can, in an ap-



propriate medium, synthesize RNA from a mixture of purines and pyrimidines, can synthesize proteins in the presence of either the purines and pyrimidines or RNA, and can form enzymes adaptively. Penicillin at a concentration of 1  $\mu\text{g. per ml.}$  inhibited the adaptive formation of  $\beta$ -galactosidase, but even at 300  $\mu\text{g. per ml.}$  had no important effect on the formation of catalase or on the enzymes involved in glycolysis. Penicillin at 30  $\mu\text{g. per ml.}$  also caused a 65 per cent inhibition of the incorporation of  $\text{C}^{14}$ -uracil into nucleic acid. The detailed papers will be awaited with interest.

*Binding of penicillin by bacteria.*—Studies with radioactive penicillin in a number of laboratories have thrown light on the kinetics of its interaction with the cell, and on the factors determining penicillin sensitivity, but as yet have provided no clue to the intimate biochemical systems affected (107 to 120).

Penicillin-sensitive strains of bacteria rapidly bound and concentrated the antibiotic, with almost complete equilibration within 1 hr. at 37°C. With bacteria as they occur in nature, the amount of antibiotic bound by bacteria from concentrations of 0.001 to 0.1  $\mu\text{g. per ml.}$  was closely related to the penicillin-sensitivity of the strain, i.e., the more sensitive the organism, the more was bound. At low concentrations of the antibiotic (0.001 to 0.01  $\mu\text{g. per ml.}$ ) penicillin was concentrated as much as 200-fold by highly sensitive strains, e.g., of *Streptococcus pyogenes*. This binding was specific in the sense that it was not observed with penicillamine or penicilloic acid. Even repeated washings removed only a small amount of this specifically bound material.

In addition to the specific binding, there was a nonspecific binding, observed equally with penicillin and its degradation products. At low concentrations the amount of nonspecific binding was negligibly small in relation to the specific binding by sensitive strains; but at high concentrations, in excess of 1  $\mu\text{g. per ml.}$ , this nonspecific binding overtook and exceeded specific binding. Contrary to earlier reports, the degree of binding was not related to the metabolic state of the organisms (115, 116); bacteria suspended in salt solution bound as much as did the same organisms in the logarithmic phase of growth. The correlation between the penicillin-sensitivity of bacteria and their binding capacity did not reflect differences in permeability, since cell-free extracts combined with penicillin in the same order as the intact bacteria, and again in relation to their penicillin-sensitivity. The correlation between sensitivity and penicillin-binding therefore reflects differences in the actual reactivity of cell components with the antibiotic (116).

Once bound, penicillin apparently has the same toxicity for all bacteria, no matter what their sensitivity to the antibiotic. Approximately 900 to 1200 molecules per cell were found to be bound without demonstrably affecting the rate of growth of *Streptococcus pyogenes*, *Diplococcus pneumoniae*, *Micrococcus pyogenes*, or *Streptococcus galactiae*. When the amounts bound ranged between 1500 to 1700 molecules per cell, there was a definite growth-inhibitory effect, and bactericidal action was observed in the range 1500 to 2400 molecules per cell (120). These results were consistent with the thesis



that bacteria normally contain an essential penicillin-vulnerable component in excess of their minimum requirement, and that the death of the cell results only when most of this component has been inactivated by its combination with the antibiotic. The foregoing relationship between penicillin sensitivity and penicillin binding applies only to bacteria as they occur in nature, and not to resistant variants produced in the laboratory by selective propagation in the presence of the antibiotic (q.v. p. 181).

Attempts to isolate and to identify the binding component have, to date, been unsuccessful. Cooper (121) has found the binding component to be associated with a lipid fraction of *M. pyogenes*, a finding not confirmed by Eagle (122). Cooper, Rowley & Dawson (109) first reported that penicillin was concentrated within the cytoplasm of *M. pyogenes* as distinct from the cell wall. With improved methods of disrupting the cell, however, Few, Cooper & Rowley (123) found a penicillin-binding component to be associated with the cell wall. Daniel & Johnson (124) have found that BAL (dimercaptopropanol) and cyanide significantly reduced the binding of penicillin by whole cells, an effect which was reversed by aeration,  $\text{Co}^{++}$  and  $\text{Mn}^{++}$ .

The relationship of penicillin binding to the adaptive formation of penicillinase is discussed in a following section.

The failure of penicillin in reasonable concentrations to kill mammalian cells is not simply a consequence of their impermeability to the antibiotic. Active penicillin could be demonstrated in two mammalian cell lines after their exposure to penicillin in tissue culture (118); but, as in the case of highly resistant bacteria, the cell components had a low order of chemical reactivity with the antibiotic. This finding poses a problem in explaining the apparent resistance of intracellular bacteria to the action of antibiotics (125) (cf. below).

*Factors modifying the bactericidal action of penicillin.*—It seems clear that the mode of action of penicillin cannot yet be described in chemical terms. There have been a number of observations relevant to its cytotoxic effect, the explanation of which must presumably await that final clarification. Perhaps the most important observation, still unexplained, is the early one that penicillin kills only actively multiplying organisms, or rather, as indicated by Chain & Duthie (126), organisms in an environment favorable for growth.

The physiological state of the bacteria, therefore, materially conditions the cytotoxic action of penicillin. The same apparently holds for a number of other antibiotics as well (cf. pg. 185). In experimental muscle infections of mice with group A streptococci (127), in an enterococcal endocarditis of rats (128), and also in an experimental nephritis of rats (128), when bacteria had accumulated in such large numbers at the foci of infection as to limit their growth, there was a marked reduction in the rate of bactericidal action, in the proportion of cures, and in the rate of healing of the lesions. The extent to which similar situations in man may depress the therapeutic

action of penicillin and other antibiotics remains to be determined. The fact that intracellular bacteria are apparently protected against antibiotics (125, 129, 130), despite the fact that at least two mammalian cells have been shown to be freely permeable to penicillin in tissue culture (118), may well depend on a similar slowing down of the growth processes of the bacteria in an unfavorable environment.

The zone phenomenon, i.e., the fact that penicillin kills some bacteria more slowly at high concentrations than at low concentrations (131, 132), remains to be explained. Gunnison, Kunishige & Jawetz (133) have made the interesting observation that neomycin also has an optimally effective concentration with *Streptococcus fecalis* or *M. pyogenes*, but only in the absence of nutrients permitting bacterial multiplication. A similarly puzzling observation is the "auto-killing" phenomenon (134),—the fact that, in agar suspensions containing large numbers of bacteria and threshold concentrations of penicillin, substances are released into the surrounding fluid which bring about the death of normally resistant organisms derived from the same strains.

The importance of the composition of the medium on penicillin action was indicated by the early observations of Schwartzman (135) that the amino acid content of the medium greatly affected the penicillin sensitivity of gram negative bacteria. Simmonds & Fruton (95, 96) found that penicillin action on an unidentified organism was enhanced when a dipeptide was replaced by its component amino acids, and suggested that penicillin might be inhibiting the synthesis of the essential peptide. Using *E. coli* as the test organism, Wainwright & Mullaney (135a) found penicillin to be much more effective with glucose in the medium than with xylose or ribose, and attributed it to the fact that the cells could metabolize glucose but not the pentoses. The possibility must be considered that at least some of the reported effects of the medium on the action of penicillin are similarly the result of effects on bacterial growth, and not directly related to the mode of action of penicillin.

The significance of the minor enhancement of penicillin action by cobalt (72, 136, 137) is difficult to assess. Faguet (137) has found cobalt to be growth-stimulatory for *M. pyogenes*; and its effect on penicillin action may be related to that phenomenon.

Parker (138) has analyzed the effect of brief exposure to penicillin on the subsequent death of the bacteria. Bacteria in an optimal medium at high pH (8.1) and at 37°C. continued to die after the penicillin had been removed from the medium; while at lower pH (6.9) and at lower temperature (25°C.) the bacteria did not lose their viability and eventually recovered.

A number of workers have studied the bizarre morphologic changes caused by the action of penicillin, including the formation of L-forms (139), and the reversion of the altered cells to normal morphology on cultivation in the absence of antibiotic (140). There have been attempts to interpret nuclear and cytoplasmic changes in terms of the locus of action of penicillin (141), but the ever-present difficulty of distinguishing between a direct effect of the

antibiotic and a secondary effect in a dying cell appears particularly cogent in this instance. The supposed sensitization of bacteria to penicillin after they had been exposed to its action *in vivo* (142) is reported to reflect an error in the bioassay procedure (143).

In general, penicillin and the host defense mechanisms appear to supplement each other in bringing about cure. In a group A streptococcal infection it has been shown that otherwise subcurative doses of penicillin suffice to effect cure in animals with a minor degree of immunity (84). The host defenses in such instances are capable of disposing of small numbers of bacteria which would otherwise have survived the action of the drug, but are incapable of dealing with large numbers. There has been no further evaluation of the antitoxic effect of penicillin, as reported by Miller & Boor (144, 145), or of its clinical significance. Quan *et al.* (146) have similarly found chlorotetracycline, chloromycetin and oxytetracycline to protect mice from the toxin of *Pasteurella pestis*.

#### STREPTOMYCIN

With streptomycin, as with penicillin, there have been a number of apparently unrelated observations bearing on the mechanism of its cytotoxic effect.

Macheboeuf (147) found that streptomycin inhibited the dephosphorylation of mononucleotides and the depolymerization of nucleic acids. Wight & Burk (148) found that it inhibited the oxygen consumption of resting cell suspensions of *E. coli* when serine, threonine, and a number of tricarboxylic acid cycle intermediates were used as substrates. Similarly, streptomycin inhibited the aerobic and anaerobic deamination of aspartic acid, and the aerobic deamination of serine. Barkulis (149) described an inhibitory effect of dihydrostreptomycin on pyruvate fermentation in *E. coli*, not observed with either resistant or dependent cells. On the other hand, streptomycin has been found to stimulate the oxygen consumption of *E. coli* with glucose, pyruvate, succinate, glutamate, malate, and glycerol as substrates (150). This was tentatively ascribed to a more complete oxidation resulting from an inhibition of assimilatory processes. Zeller (151) found that the diguanidine grouping of streptomycin inhibited a diamine oxidase of mycobacteria. Several purine and pyrimidine derivatives were found to reverse the effect of streptomycin, but not that of dihydrostreptomycin, on *Aerobacter aerogenes*; whereas L-phenylalanine reversed the action of dihydrostreptomycin, but not that of streptomycin (152).

A relationship to pantothenic acid synthesis was indicated by the fact that with  $\beta$ -alanine in the medium as the only source of pantothenic acid, streptomycin caused a marked inhibition of growth of the relatively resistant *Saccharomyces fragilis*; but there was no inhibition if pantothenic acid, as such, was added to the medium (153). It would be of interest to determine if this relationship holds with more sensitive organisms. A number of B vitamins have been found to reverse the cytotoxic effect of streptomycin (154).

With many of these reports, the usual question arises as to whether one is dealing with a direct effect of the antibiotic on the metabolic activity under investigation, or whether instead these are secondary effects in a dying cell. Thus, in relation to Zeller's observation of an inhibition of diamine oxidase activity, Geronimus observed no such inhibition in cell-free preparations (155).

The mechanism of streptomycin action which has been most forcefully advanced is that proposed by Umbreit and his co-workers, that streptomycin inhibits the condensation of pyruvate and oxaloacetate (156) to a seven-carbon compound which does not operate through citrate (157). More recently, a seven-carbon compound has been shown to be formed by *E. coli* in the presence of pyruvate and oxaloacetate, and identified as the 2-phospho-4-carboxyadipic acid (158). The thesis that streptomycin affects the terminal oxidation of glucose has, however, been questioned by Paine & Clark (159). A strain of *M. pyogenes* which, under anaerobic conditions, simply reduced pyruvate to lactate was readily killed by streptomycin in both the resting and growing state. Further, it was found (160) that the antibiotic may inhibit, stimulate, or have no effect on oxygen uptake, depending on the carbon source on which the bacteria were grown and the substrate. The killing action of streptomycin was definitely related to the metabolic activity of the organisms but not to its effects on respiration (161). In keeping with the observations of Fitzgerald *et al.* (162), Paine & Clark (160) suggest that streptomycin, in inhibiting respiration, may interfere with the formation of the appropriate adaptive enzyme. The observations of Vignais & Vignais (163) are pertinent in this connection. As suspensions of *E. coli* passed through the logarithmic phase of growth into the stationary phase, they were found to catabolize acetate more and more rapidly, presumably due to the synthesis of the enzymatic systems concerned. If streptomycin was added for 1 hr. at the end of the growth period and the cells were then revived by washing, there was only a slight decrease of acetate oxidizing capacity; but if it was added during the first half of the exponential phase of growth, acetate oxidation was almost completely inhibited. They suggest that it may interfere with the synthesis of the enzymatic systems responsible for continued oxidation.

It is difficult to assess the significance of the bacterial agglutination often caused by streptomycin (164, 165). Lysozyme-sensitive bacteria were not agglutinated by streptomycin; indeed, the latter inhibited the clarification of bacterial suspensions by lysozyme, chiefly by preventing nuclear dissolution (166). It has been suggested that this agglutinating action depends on a reaction with nucleoproteins analogous to the precipitation of viral deoxyribonucleic acid by streptomycin reported by Cohen (167).

*The susceptibility of resting bacteria to streptomycin.*—There has been a good deal of contradictory evidence as to whether streptomycin affects resting bacteria or whether, as in the case of penicillin, it affects only actively growing organisms. Hamre, Rake & Donovick (168) found that resting cells of *Klebsiella pneumoniae* were rapidly killed by streptomycin; and *Serratia*

was found to be inhibited even when growth was retarded by the omission of phosphate from the medium (169). Similarly, streptomycin inhibited respiration in resting cell suspensions (170, 171, 172).

In contrast to these findings, however, Middlebrook & Yegian (173) had found that *Mycobacterium tuberculosis* was not affected after 72 hr. exposure in salt solution at 20  $\mu$ g. per ml., but that 99 per cent of the organisms were killed in 24 hr. in Dubos medium. Similar findings were obtained by Garrod (174). Bonifas & Novel (175) found that 10 times as much streptomycin was required to kill *E. coli* in the stationary phase of growth as sufficed with organisms in the latent or logarithmic phase. The survivors relatively resistant to the drug in the stationary phase of the culture were, however, not similarly resistant when subcultured. Rosenblum & Bryson (155) also found that the killing of *E. coli* by streptomycin occurred only when the organism was in a medium suitable for growth, and that insignificant killing was observed in glucose-free medium. The metabolism of as little as 20  $\mu$ g. of glucose per ml. initiated the cytotoxic effect.

In summary, there is reason to believe that some of the metabolic effects of streptomycin are not necessarily related to its cytotoxic action; that with some strains the cytotoxic effect is not materially affected by the state of metabolic activity and the composition of the medium, while with others, notably *E. coli*, it is profoundly modified. The inhibition of streptomycin activity by the addition of relatively high concentrations of pyruvate or fumarate to the medium, and the failure of these compounds to affect its cytotoxic action for *Aerobacter aerogenes* and *Micrococcus pyogenes* (175a) are some of many observations which do not fit neatly into any simple hypothesis.

*Miscellaneous observations.*—Lightbown (176) has demonstrated that *Pseudomonas aeruginosa* produced a highly active antagonist for streptomycin and dihydrostreptomycin. With certain organisms 0.1  $\mu$ g. per ml. antagonized 10  $\mu$ g. of streptomycin per ml. The antagonist has since been identified as a mixture of 4-hydroxy-quinoline-N-oxides, with side chains of 7, 8, and 9 carbons (177). The significance of this interesting observation with respect to the mode of action of streptomycin has not yet been clarified.

Streptomycin was found not to affect the germination of clostridia, and was not sporicidal (178).

*In vivo*, streptomycin has been found to cause a greater phagocytosis of *Mycobacterium tuberculosis* by guinea pig leukocytes due to an effect on the organisms rather than the leukocytes (77, 78, 79, 79a). Similar observations have been made with a number of bacterial strains (80). Such phagocytosed organisms were relatively resistant to the drug, but when the phagocytes died, the bacteria became vulnerable to the antibiotic (77). The concentration of streptomycin necessary to inhibit the growth of tubercle bacilli inside guinea pig or rabbit macrophages may be 100 times that necessary in a Tween albumin medium (179). However, preliminary studies with radioactive streptomycin indicate that the antibiotic does get into certain mammalian

cells in active form (122). The relative resistance of intracellular *M. tuberculosis* to the drug may therefore not necessarily be related to the impermeability of the cell, but rather to the metabolic phase of the organisms, as discussed in a previous section.

The electron microscopic changes produced by the action of streptomycin on *M. tuberculosis* have been studied by several workers (180, 181, 182). Linz & Arnaud (182a) found that streptomycin actually combines with both *Micrococcus lysodeikticus* and *Mycobacterium tuberculosis*, the amount of streptomycin bound after exposure to a concentration of 1000  $\mu\text{g. per ml.}$  for 4 hr. amounting to 1.28  $\mu\text{g. per 50 mg.}$  of bacteria. It should, however, be noted that only free streptomycin was measured, and that this value is not necessarily a measure of the total bound antibiotic.

The cytotoxic action of streptomycin is not limited to bacteria. Streptomycin caused the elimination of chloroplasts from *Euglena gracilis* without affecting the growth of the organism, provided that adequate carbon and energy sources were supplied. Both proliferating and nonproliferating cells were equally susceptible (183). Provasoli, Hutner & Schatz (183) cite observations by Von Euler & Bracco to the effect that when seeds were allowed to germinate in the presence of streptomycin, there were chlorophyll deficiencies in the leaves of the resulting seedlings. Streptomycin in concentrations of 0.1  $\mu\text{g. per ml.}$  has been found to inhibit the growth of *Avena coleoptile* sections. This inhibition, unlike the bactericidal action on *E. coli*, was reversed by  $\text{Mn}^{++}$  (184).

#### CHLORAMPHENICOL

*Chloramphenicol and amino acid metabolism.*—There have been several reports that chloramphenicol affects the amino acid metabolism of sensitive bacteria, and that its cytotoxic action is reversed by amino acids. Thus, Woolley (185) found that its growth-inhibitory effect at a concentration of 1  $\mu\text{g. per ml.}$  on a strain of *E. coli* was completely reversed by the addition of phenylalanine to the medium at a concentration of 500  $\mu\text{g. per ml.}$  No reversal was obtained at somewhat higher concentrations of antibiotic (2  $\mu\text{g. per ml.}$ ). Similar results were obtained with tyrosine and tryptophan. With *Lactobacillus casei*, only phenylalanine was effective in reversing chloramphenicol inhibition. As with *E. coli*, the reversal occurred only within narrow concentrations of the antibiotic. Similarly, Mentzer *et al.* (186) found that glycine antagonized the inhibition of *E. coli* by chloramphenicol, while aspartic acid and serine markedly potentiated its action. Tryptophan had no effect. Truhaut *et al.* (187) have shown that the antibiotic inhibited the synthesis or breakdown of tryptophan or its precursors (anthranilic acid, indol) by *Salmonella typhosa*. As the tryptophan concentration of the medium was increased through the range of 5 to 500  $\mu\text{g. per ml.}$ , the amount of chloramphenicol necessary to inhibit growth rose from 3  $\mu\text{g. per ml.}$  at a tryptophan concentration of 5  $\mu\text{g.}$ , to 15  $\mu\text{g. per ml.}$  at a tryptophan concentration of 500  $\mu\text{g. per ml.}$



In contrast to these reports, Molho & Molho-Lacroix (188) reported little or no reversal of the chloramphenicol inhibition of *E. coli* by phenylalanine. Slight reversal was obtained with glycylphenylalanine, p-nitrophenylalanine and glycyl-p-nitrophenylalanine. These authors believe that chloramphenicol, since it is an amide, may interfere with the metabolism of a dipeptide in the cell, and feel it more logical to look for reversal by these compounds rather than by the amino acids. Gale & Paine (189, 190) reported that chloramphenicol had little or no effect on glutamic acid assimilation by *M. pyogenes*, even at concentrations of chloramphenicol as high as 1.5 mM.

Bergmann & Sicher (191) reported the reversal of inhibition in *E. coli* by tyrosine, tryptophan, and indole but not by anthranilic acid. They concluded that chloramphenicol interferes with the synthesis of indole from anthranilic acid via tryptophan.

In terms of specific metabolic effects, Bernheim & DeTurk (192) found that chloramphenicol inhibited the oxidation and, to a lesser extent, the deamination of phenylalanine, tyrosine, and phenylserine by *Pseudomonas aeruginosa*. Succinate oxidation was also inhibited if the antibiotic was added before, but not after, the substrate. During normal oxidation of phenylalanine no ammonia accumulated, indicative of its assimilation; and similar partial assimilation was observed during the oxidation of tyrosine and phenylserine. This oxidative assimilation of ammonia was inhibited by antibiotics, and it was suggested that the antibiotics interfered with the formation of compounds essential for ammonia assimilation.

Smith *et al.* (193) have reported that bacteriostatic concentrations of chloramphenicol inhibited the hydrolysis of tributyrin by bacterial and crystalline liver esterase. At lower concentrations there was some enhancement of esterase activity. Vincent & Parant (194) reported that the antibiotic did not inhibit brain or serum cholinesterase. In *E. coli*, chloramphenicol, oxytetracycline, chlortetracycline, and erythromycin inhibited the oxidation of glutamic acid at concentrations which were not significantly bactericidal over the period of observation (195). Penicillin was without effect, and streptomycin caused an increased oxidation.

Bone marrow incubated with folic acid has been found to synthesize "hemofolin," a growth factor for *Leuconostoc citrovorum* (196). This compound is apparently related to folic acid, and proved capable of reversing the growth-inhibitory effect of chloramphenicol on *L. citrovorum*.

*Effects on protein and nucleic acid synthesis.*—There are several reports indicating that chloramphenicol drastically inhibits protein synthesis in sensitive bacteria. Earlier work (197, 198) showed that the antibiotic inhibited the synthesis of adaptive enzymes mediating the oxidation of lactose and the phosphorylation of gluconic acid in *E. coli*. The antibiotic had no effect on these reactions in cells which had been previously adapted to lactose and gluconate oxidation. Gale and his co-workers (189, 190, 199) showed that chloramphenicol at concentrations of 1 to 5  $\mu$ g. per ml. inhibited protein synthesis by *M. pyogenes* as much as 90 per cent. These amounts of



chloramphenicol were growth inhibitory. Fermentation, respiration, free glutamic acid accumulation, and nucleic acid synthesis were affected only by much higher antibiotic levels. The incorporation of isotopically labelled phenylalanine by washed suspensions of *M. pyogenes* was competitively inhibited by p-chlorophenylalanine and noncompetitively inhibited by chloramphenicol (92, 93). The incorporation of glutamic acid was much less sensitive to chloramphenicol inhibition than that of phenylalanine. With both amino acids the inhibition by chloramphenicol was much greater in a medium containing a full complement of amino acids and normally permitting active protein synthesis.

Wisseman and his co-workers (200) found that chloramphenicol at bacteriostatic concentrations inhibited the assimilation of ammonia by resting *E. coli* by 80 per cent, while oxygen consumption, with glycerol as substrate, was inhibited in the presence of ammonia but not in its absence. Further, protein synthesis was abruptly stopped in growing *E. coli* cultures when chloramphenicol was introduced into the medium, while RNA and DNA syntheses were unimpeded. Antibiotically inactive isomers of chloramphenicol were ineffective in inhibiting protein synthesis. Since chloramphenicol is the D(-)threo isomer and inhibited the formation of proteins from L-amino acids, Hahn *et al.* (201) thought it possible that the L-isologues of chloramphenicol might conversely inhibit D-polypeptide formation. The L(+)erythro isomer, though with no effect on growth, did in fact inhibit D(-)polypeptide formation; while the D(-)threo isomer (chloramphenicol) inhibited growth but was without effect on D(-)peptide synthesis.

In keeping with the thesis of an inhibition of protein and nucleic acid synthesis, it is pertinent to note that bacteriostatic concentrations of chloramphenicol inhibited the intracellular growth of T-1 coliphage (202). The drug had no direct anti-phage activity, and did not inhibit adsorption of the phage on *E. coli* B, but inhibited phage maturation at any stage during the latent period. Bacteriostatic concentrations added early in the latent period inhibited both the formation of new phage particles and lysis. This early inhibition was partially reversible by diluting out the chloramphenicol. If the antibiotic was added late in the latent period, lysis occurred at the usual time but there was no increase in phage from the time the drug was added.

Brown (203) has reported the elimination of "kappa" particles from killer strains of *Paramecium* by high concentrations of chloramphenicol.

In Ehrlich carcinoma cells, the incorporation of glycine-2-C<sup>14</sup> into both protein and nucleic acid was found to be inhibited by chloramphenicol (204). The L(+) threo isomer inhibited glycine incorporation into purines, but even at concentrations of 2000  $\mu$ g. per ml. inhibited incorporation into proteins only slightly. In lymphosarcoma 6C3HED, both isomers were equally effective in inhibiting the incorporation of glycine into proteins and purines. Its incorporation into guanine was inhibited much more than that into adenine.

Chloramphenicol did not inhibit polysaccharide synthesis in *Neisseria perflava* (205), and Smith (206) has listed a number of enzymatic systems

unaffected by the antibiotic. The striking photographs of Bergersen (207) indicate that chloramphenicol affects nuclear division in *E. coli*.

*Chloramphenicol metabolism by bacteria.*—Chloramphenicol is susceptible to enzymatic attack at several loci. Smith & Worrel (208, 209) in particular have shown that *E. coli*, *Bacillus mycoides*, *B. subtilis* and *Proteus vulgaris*, all sensitive to the drug, reduced the nitro group of the antibiotic to the corresponding arylamine, which was antibiotically inactive. Succinate, formate, and lactate increased the amount of arylamine formed, indicating that various dehydrogenases were linked with the reductase complex. The free base formed by hydrolysis of the amide group of the antibiotic was reduced several times faster than the parent compound; and the fact that cysteine favored the reduction of chloramphenicol was attributed to its stimulation of this hydrolysis. The ease of reduction was influenced by the kind of halogen atom on the dichloroacetamido portion of the molecule, the arrangement of the atoms in the aliphatic portion, and the presence of free or acetylated hydroxyl groups; but the configuration of the chloramphenicol molecule had little influence. Smith and his co-workers (208) have described the oxidation of the secondary hydroxy group of chloramphenicol by various bacterial species, and the splitting of the molecule between the first and second carbons of the propanediol residue. Many of the fragments so formed were further oxidized.

Egami, Ebata & Sato (210) presented evidence that an enzyme in cell-free *Streptococcus hemolyticus* extracts, which reduced chloramphenicol and other nitro compounds anaerobically, would also reduce inorganic nitrite. Saz and collaborators (198, 211) extracted an enzyme complex from *E. coli* E-26 which reduced chloramphenicol and some other aromatic nitro compounds. For complete reduction to arylamine this system required L-cysteine (D-cysteine was approximately 25 per cent as active as the L-isomer), DPNH (dihydrodiphosphopyridine nucleotide), and a divalent cation; and preliminary evidence indicated a requirement for a conjugated flavin. Successive reduction of the nitro group through nitroso and hydroxylamino derivatives to the amino compound was postulated. Because there was no mutual inhibition between aromatic nitro compounds and inorganic nitrite, and because a number of inhibitors did not similarly affect the two reactions, it was concluded that in *E. coli* the reduction of organic nitro and inorganic nitrite groups were not mediated by the same enzyme complex.

In a later publication from Egami's laboratory, Yamashita (212) extracted a cell-free nitroreductase from the halotolerant organism, *Bacillus pumilus*. It was postulated that the "nitroreductase" proceeded through nitrosoreductase, hydroxylaminoreductase, etc., and that the system included a hydrogen donor, dehydrogenase, an intermediary hydrogen carrier (dyes), and finally the "nitroreductase" complex. In this extract, inorganic nitrite reductase and nitroreductase did not appear to be identical.

*Specificity of the chloramphenicol molecule for antibiotic activity.*—Smith (213) has studied the effect on the growth of *E. coli* of the decomposition

products of chloramphenicol formed as a result of bacterial activity, and their ability to reverse the growth-inhibitory action of the antibiotic. The p-nitrophenyl group has been found to be relatively nonspecific in that some antibiotic activity remained after its replacement by p-chlorophenyl, p-dichloroacetyl-amido and other groups (214). However, replacement of the  $-NO_2$  group by cyano, carbamoyl, ethoxycarbonyl, and thiazolyl groups yielded compounds less than 1/10 as active as chloramphenicol (215). Similar results were obtained upon replacing the dichloroacetamido grouping by a ureido group. Maxwell & Nickel (216) found that of four isomers of chloramphenicol, the L-erythro isomer was only 1 to 2 per cent as active as the D-threo (chloramphenicol) in inhibiting growth of *E. coli*, and the L-threo and D-erythro isologues were less than 0.4 per cent as active. The D-threo and the L-erythro isomers at concentrations of 1 to 10  $\mu\text{g. per ml.}$  and 100  $\mu\text{g. per ml.}$ , respectively, caused inhibition of protein synthesis, but no inhibition of nucleic acid synthesis as measured by  $S^{35}$  and  $P^{32}$  incorporation studies. The L-threo and D-erythro isomers were inactive. The results of Wisseman *et al.* concerning the effect of chloramphenicol on protein synthesis have been described in a preceding section.

#### THE TETRACYCLINES

It is generally assumed that chlortetracycline, oxytetracycline, and tetracycline, because of their close chemical relationship (44, 45), have similar modes of action. Tetracycline is the parent compound; chlortetracycline possesses, in addition, a chlorine atom in the unsaturated (D) ring, and oxytetracycline, a hydroxyl group in the (B) ring. Their antibiotic spectra are similar (217), and almost complete cross resistance has been reported by many investigators (217a). Nevertheless, there have been reports of significant qualitative as well as quantitative differences in their inhibitory effects, and the possibility remains that there may be important differences in their modes of action.

As described below, the tetracyclines have been reported to affect oxidation and fermentation in a number of bacterial species; there is strong evidence of a derangement of protein synthesis, and of reactions involved in protein synthesis; and there is evidence also of an interference with cellular and enzymatic processes by virtue of the affinity of these compounds for inorganic cations. Which, if any, of these three general effects is related to the cytotoxic action remains to be determined.

*Effects on oxidation and fermentation.*—There have been several reports indicating that the tetracyclines inhibit oxidations by whole cells of different bacteria. In interpreting these observations one is again faced with the difficulty of distinguishing between a primary effect of the antibiotic, and a secondary effect in a dying cell. McCullough & Beal (218) reported that, in *Brucella*, chlortetracycline at concentrations of 250 to 500  $\mu\text{g. per ml.}$  inhibited the oxidation of glucose 40 to 87 per cent, the endogenous respiration by 40 to 55 per cent, and the oxidation of pyruvate, D-fructose, D-xylose,

and D-trehalose by 64, 54, 33 and 52 per cent, respectively. There was no reversal of inhibition by the addition of 100 times the usual amount of thiamin, nicotinic acid, and biotin (essential growth factors), and the effects persisted after removal of the external chlortetracycline. Similarly, oxytetracycline at 400  $\mu\text{g.}$  per ml. caused a 40 per cent inhibition of glucose oxidation by *Brucella* strains. Osteux, Laturaze & Burck (219) reported that chlortetracycline at concentrations of  $5 \times 10^{-4}$  M inhibited the oxidation of tricarboxylic acid cycle intermediates by whole cells of *E. coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris*. The endogenous respiration was also strongly inhibited. Wong, Barban & Ajl (220) confirmed these observations for both chlortetracycline and oxytetracycline, and found that acetate oxidation was particularly sensitive to the inhibitory effect of the antibiotics. Mulli, Uhlenbroock & Ludwig (221) reported that  $10^{-7}$  M chlortetracycline inhibited citrate breakdown by beef liver preparations, and that preliminary treatment of the antibiotic with alkali destroyed its activity. In this system L-cysteine acted as a chlortetracycline antagonist. Since chlortetracycline has quinone groups and thus reacts chemically with sulfhydryl groups, and since the oxidation of the tricarboxylic acid cycle intermediates requires sulfhydryl-containing Co A as a cofactor, these workers suggest the antibiotic may be blocking Co A activity. Crema & Baroli (222) reported that oxytetracycline was almost without effect on the respiration of *Staphylococcus curcio* if the organism was in the resting state, while as little as 0.1 to 1  $\mu\text{g.}$  per ml. inhibited respiration by 50 to 60 per cent at the beginning of the log phase.

Oxytetracycline and chlortetracycline were found by Bernheim & deTurk (192) to inhibit the oxidation and, to a lesser extent, the deamination of phenylalanine, tyrosine, and phenylserine. Both antibiotics inhibited the reassimilation of the  $\text{NH}_3$  produced during the oxidation of tyrosine and phenylserine and it was suggested that the antibiotic interfered with formation of compounds necessary for that assimilation.

Porro & Soncin (223) reported that chlortetracycline and oxytetracycline at 3  $\mu\text{g.}$  per ml. inhibited the oxidation of acetoacetate by *E. coli* by 40 per cent. Chloramphenicol and dihydrostreptomycin inhibited the oxidation by 20 per cent and the inhibition was slower. Penicillin was noninhibitory. Glutamic acid oxidation was also inhibited (195).

*Effects on protein and nucleic acid synthesis.*—There are several reports which indicate that the tetracyclines cause a marked derangement of cellular mechanisms leading to protein synthesis and nucleic acid formation. Hahn & Wisseman (197) showed that chlortetracycline and oxytetracycline, as well as chloramphenicol and nitroacridine 3582, inhibited the formation in *E. coli* of an induced enzyme complex mediating the oxidation of lactose. The oxidation of lactose by cells previously adapted to lactose was unaffected. The authors postulated impairment by the antibiotics of the protein synthesizing mechanisms. Bernheim (224) studied the adaptive oxidation of benzoic acid by whole cell preparations of *P. aeruginosa*. Incubation of washed cells with succinate, malate, fumarate, pyruvate, acetate, or glucose

caused the oxidation of these substrates and the formation of an unknown compound which could be utilized for the subsequent synthesis of the benzoic acid oxidizing complex. Drugs which inhibit benzoic acid oxidation when added to the bacterial suspension before the addition of succinate could be inhibiting either the formation or utilization of this compound. On the other hand, drugs added after succinate oxidation would be affecting only the utilization of the compound. Based on these considerations, Bernheim (224) reported that oxytetracycline and chlortetracycline inhibited equally both the utilization and formation of the unknown compound; streptomycin was more effective in preventing its formation; and chloramphenicol and arsenite in preventing its utilization. It should be mentioned that the antibiotic concentrations used were within bacteriostatic limits and that these concentrations had no effect on the oxidation of succinate *per se*.

Gale and co-workers (189, 190, 199) have studied the effect of chlortetracycline and oxytetracycline on nucleic acid synthesis, protein synthesis, and free amino acid accumulation by *M. pyogenes*. With both antibiotics, concentrations in the range 0.2 to 0.4  $\mu\text{g. per ml.}$  (even lower than the growth-inhibitory level of 0.5 to 1  $\mu\text{g. per ml.}$ ) inhibited protein synthesis, measured by the incorporation of isotopically labelled glutamate into protein. Much higher concentrations (50 to 500  $\mu\text{g. per ml.}$ ) were required to inhibit either the synthesis of nucleic acid, the accumulation of free glutamate, or glucose fermentation.

Samuels (225) has reported that oxytetracycline and chlortetracycline, as well as eight other antibiotics, had no effect on glutathione synthesis by suspensions of *Proteus*. Oxytetracycline in high concentrations inhibited transpeptidation and the formation of new peptides by kidney preparations.

Chlortetracycline in low concentrations inhibited the synthesis of ribose and deoxyribose nucleic acid by *Lactobacillus casei* (226). Folic acid and vitamin B<sub>12</sub> were reported to prevent this inhibition. Conversely, bacteriostatic concentrations of chlortetracycline and oxytetracycline, as well as chloramphenicol, streptomycin, and penicillin have been found to inhibit folic acid and vitamin B<sub>12</sub> synthesis in *E. coli*, and in proportion to the concentration used (227). Calet *et al.*, however, found chlortetracycline to have no effect on vitamin B<sub>12</sub> accumulation in the liver of the rat (228).

*Effect of cations on the activity of the tetracyclines.*—Recent studies indicate that the tetracyclines may inhibit various enzymatic systems and the growth of susceptible organisms by interfering with inorganic ion metabolism.

(a) Mammalian systems.—There have been several reports indicating that chlortetracycline has an inhibitory effect on oxidation and phosphorylation in mammalian tissue. Loomis (229) reported that chlortetracycline uncoupled oxidative phosphorylation in normal mitochondria. The antibiotic was without effect on oxygen consumption but, at concentrations greater than 150  $\mu\text{g. per ml.}$ , drastically inhibited orthophosphate uptake. Similarly, Brody & Bain (230) reported that chlortetracycline uncoupled

oxidative phosphorylation in rat liver and brain. On the other hand, Van Meter & Oleson (231) found that chlortetracycline, in the absence of citrate, completely inhibited the respiration of whole rat liver homogenates within 10 min. In the presence of citrate, the inhibitory effect on oxygen consumption was delayed for 30 to 60 min. Subsequently, Van Meter *et al.* (231a) confirmed the inhibition of oxidative phosphorylation by chlortetracycline in rat liver mitochondria and found that it inhibited oxidations carried out in the presence of  $Mg^{++}$ , low phosphate concentrations and ATP (adenosinetriphosphate). The addition of  $Mg^{++}$  at levels at least twice those of the antibiotic reversed the inhibition. In the same system, Brody, Hurwitz & Bain (232) found that all three tetracyclines uncoupled oxidative phosphorylation and inhibited octanoate oxidation. In both reactions there was a requirement for added  $Mg^{++}$ , and the inhibitions were reversed at higher  $Mg^{++}$  levels. Of the three antibiotics, chlortetracycline was least readily reversible by  $Mg^{++}$ , reversal requiring  $Mg^{++}$ : chlortetracycline ratios of 20 to 50:1. A  $Mg^{++}$  dependent yeast hexokinase was also inhibited by chlortetracycline, but only at low enzyme levels (1 to 2 units per ml.). At enzyme concentrations of 30 units per ml., even high concentrations of chlortetracycline caused no inhibition.

(b) Bacterial systems.—There have been several reports indicating that the tetracyclines inhibit both bacterial growth and certain enzymatic reactions in cell-free extracts by virtue of their ability to chelate or otherwise combine with essential cations. Saz and collaborators (198, 211) have reported that chlortetracycline, in concentrations which are bacteriostatic and sub-bacteriostatic, strongly inhibited a cell-free organic nitroreductase extracted from *E. coli*. This enzyme complex reduced chloramphenicol, *p*-nitrobenzoic acid, and other aromatic nitro compounds to the corresponding arylamines. Partial resolution of the complex indicated that the reduction was linked to malic dehydrogenase. When L-malate and DPN were used as hydrogen donor system, chlortetracycline was fully active in inhibiting the reductase but was only weakly inhibitory when DPNH in concentrations greater than chlortetracycline was used in place of malic dehydrogenase system. It was found that  $Mn^{++}$  (and  $Mg^{++}$  in higher concentrations) was essential for DPNH formation. More specifically, the cation may activate an oxaloacetic carboxylase present in the extracts. This enzyme, by mediating the decarboxylation of the oxaloacetic acid, formed by malic dehydrogenase, shifted the equilibrium and permitted the formation of significant amounts of DPNH (91).

That  $Mn^{++}$  was essential for reductase activity was shown by the loss of activity when the extract was dialyzed against versene or cyanide. Activity was completely restored by the addition of  $Mn^{++}$ . Further, the chlortetracycline inhibition of nitroreductase was reversed by  $Mn^{++}$  in concentrations 20 to 30 times greater than the antibiotic. Albert (233) had found that chlortetracycline and oxytetracycline possessed similar chelating properties for various cations including Mn. In the nitroreductase complex, however,



chlortetracycline was far more effective than either oxytetracycline or tetracycline. A number of chlortetracycline degradation products with little or no antibiotic activity inhibited arylamine formation. The bacterial cell was shown to be relatively impermeable to several of these compounds; and their weak antibiotic activity is perhaps referable to this fact. These degradation products may not inhibit the reductase at the same locus as does chlortetracycline, since the inhibition was not reversible by cations. Further, nitroreductase extracts of chlortetracycline-resistant cells which were resistant to the antibiotic were inhibited by these degradation products (91).

Soncin (234) reported that chlortetracycline and oxytetracycline inhibited glycolysis, respiration, and multiplication of *E. coli*.  $MgCl_2$  in molar concentrations 50,000 times greater than the two antibiotics reversed the inhibition of all three functions.

Weinberg (235) has investigated in detail the effects of cations on the oxytetracycline inhibition of *P. aeruginosa*.  $Na^+$  and  $K^+$  slightly enhanced its activity whereas  $Fe^{++(+)}$ ,  $Mg^{++}$ , and  $Mn^{++}$  were inhibitory. It required 2000 molecules of  $Mg^{++}$  to reverse one molecule of added oxytetracycline, but only 47 to 141 molecules of  $Fe^{++}$ .  $MoO_4^{--}$  and  $Mn^{++}$  were less effective than  $Mg^{++}$  and  $Fe^{++(+)}$ , and  $Co^{++}$ ,  $Cn^{++}$ ,  $Ca^{++}$  and  $Al^{+++}$  were ineffective. Metal complexing agents such as citrate, oxalate, tartrate and versene did not enhance oxytetracycline activity. Weinberg subsequently found (236) that  $Mg^{++}$  reversed the toxicity of oxytetracycline toward a strain of *Bacillus subtilis*, a colorless alga, and a *Micrococcus*.  $Al^{+++}$  and  $Fe^{++(+)}$  reversed its effect on the alga, while  $Ca^{++}$  reversed its effect on the *Micrococcus*. Significantly, *P. aeruginosa* cells which had been inactivated by exposure to oxytetracycline for one hour, and then washed free of antibiotic, could be reactivated by plating in  $Mg^{++}$ , and higher concentrations were more effective than lower. In sharp contrast to the foregoing observations, Kawakami (237) has reported that heavy metals slightly potentiated, rather than inhibited, the action of chlortetracycline and oxytetracycline on a *Staphylococcus*.

In keeping with most of the observations on the tetracyclines, Newton (238) was able to reverse the polymyxin inhibition of *P. aeruginosa* by the addition of  $Mg^{++}$ ,  $Mn^{++}$ ,  $Ca^{++}$  and  $Fe^{++(+)}$  to the medium. To reverse one molecule of polymyxin 400 atoms of  $Mg^{++}$  were needed. However, this effect was ascribed to a competition between the drug and the cations for absorptive sites in or on the cell, rather than to chelation.

*Reversal of tetracycline inhibition by various products.*—A number of natural products have been reported to reverse the activity of the tetracyclines. Riboflavin, folic acid, and pyridoxine added to the medium at 0.1  $\mu g.$  per ml., and thiamin and p-aminobenzoic acid in concentrations of 10 and 100  $\mu g.$  per ml., respectively, were found to inhibit the action of oxytetracycline on *M. pyogenes* (239). Biotin and pantothenic acid were without effect. Cells grown with optimal amounts of folic acid and pyridoxine for three generations were then found to be relatively resistant to the anti



biotic, even in the absence of added vitamin (239). The inhibition of *E. coli* by chlortetracycline and oxytetracycline was reversed by indol, tryptophan, phenylalanine, and tyrosine, but not by anthranilic acid. However, anthranilic acid plus methionine plus vitamin B<sub>12</sub> together reversed the activity of the two antibiotics (240), as did ATP (240a, 240b, 240c).

Wacker (241) has reported the competitive reversal of the oxytetracycline inhibition of *E. coli*, but not that of chlortetracycline, by acid hydrolyzed casein or pancreatin. The effective material had a low Rf in butanol-H<sub>2</sub>O, and was also present in yeast extract.

Foster & Pittillo (242) have similarly reported that in the presence of certain complex organic supplements such as Basamine, Difco peptone, Wilson Liver B, and Difco Brain Heart Infusion at concentrations of 100 to 1000 µg. per ml., the inhibitory concentrations of penicillin, streptomycin, chlortetracycline, oxytetracycline, and chloramphenicol for *E. coli* B were increased 4- to 8-fold. Riboflavin, biotin, thiamin, pyridoxine, and pyridoxamine, folic acid, inosine, *p*-aminobenzoic acid, and pantothenate were identified as among the active factors in reversing the effects of chlortetracycline, while vitamin B<sub>12</sub> and nicotinic acid were inactive. All the amino acids tested showed partial reversal, while the addition of glycine at 80 µg. per ml. caused an 8-fold increase in the inhibitory concentration of chlortetracycline. On the other hand, the oxytetracycline inhibition of *E. coli* B was not reversed by any of the compounds tested.

The results with other organisms were comparable to those obtained with *E. coli* B except that, in the case of *Bacillus globigii*, glycine and inosine were inactive. With a riboflavinless mutant of *B. subtilis*, higher concentrations of added riboflavin were required to reverse the antibiotic action; similarly, when riboflavin synthesis was inhibited in another strain, the organisms became more sensitive to the antibiotic. It is difficult to assess the significance of the latter observations with respect to the mode of action of the tetracyclines. Thus, with *E. coli* B, lumichrome was as effective as riboflavin in reversing chlortetracycline. Further, riboflavin synthesis was shown to be inhibited by both chlortetracycline, which was reversed by riboflavin, and oxytetracycline, which was not reversed.

Mannino & Pipitone (227) reported that chlortetracycline, oxytetracycline, penicillin, streptomycin, and chloramphenicol, in bacteriostatic concentrations all inhibited vitamin B<sub>12</sub> and folic acid synthesis by *E. coli*. They suggest that these results may explain some of the data of Foster & Pittillo, since reversal may be obtained by adding the end products of blocked reactions. On the other hand, Colet *et al.* (228) found that chlortetracycline had no effect on the accumulation of vitamin B<sub>12</sub> in rat liver.

In *L. casei* chlortetracycline inhibited the synthesis of both pentose and desoxypentose nucleic acids (226). Folic acid and higher concentrations of vitamin B<sub>12</sub> protected against this effect of the antibiotic.

Osteux *et al.* (243, 244) showed that chlortetracycline, oxytetracycline,

chloramphenicol, and penicillin inhibited the dissimilation of pyruvic acid to acetic acid,  $\text{CO}_2$  and  $\text{H}_2$  by *Clostridium welchii*. This inhibition was reversed by high concentrations of biotin.

Sloane (245) has reported that chlortetracycline inhibited the hydroxylation of aniline to *p*-aminophenol by *M. tuberculosis* 607. The inhibition was partially reversed by a compound formed upon incubation of the organism with *p*-aminobenzoic acid. This reversing compound had the empirical formula  $\text{C}_{14}\text{H}_{24}\text{N}_2 \cdot 1/2\text{H}_2\text{O}$ .

*Miscellaneous effects of the tetracyclines.*—Oxytetracycline at bacteriostatic levels, and chlortetracycline at somewhat higher concentrations, were found by Miura *et al.* (246) to inhibit strikingly (>99 per cent) the incorporation of  $\text{P}^{32}$  into nucleic acids of *M. pyogenes*, and to a lesser extent (60 per cent) its incorporation into acid-labile organic phosphorous compounds ( $\Delta 7\text{P}$ ). The time relationships are discussed in a preceding paper (247).

Free coliphage T3 was found by Altenbern (248) to be unaffected by chlortetracycline at concentrations of 3 to 20  $\mu\text{g}$ . per ml. However, preincubation of *E. coli* B with the antibiotic reduced the absorption of added phage from a normal value of 44 to 11 per cent. The treated cells regained their ability to absorb the phage upon resuspension in antibiotic-free medium. Phage growth in infected cells was completely prevented at levels of chlortetracycline which inhibited cell multiplication. If the antibiotic was added 5 to 15 min. after infection with phage, the latent period was reduced and the lysis of some infected cells was accelerated. There was indication also that the phage-infected bacteria were killed more readily by chlortetracycline than normal cells; and cationic surface-active agents promoted the death of the phage-infected, chlortetracycline-treated bacteria.

Several workers (249, 250, 251) have found that oxytetracycline and chlortetracycline can be either stimulatory, inhibitory, or without effect on a variety of microorganisms, depending on the composition of the medium. Indeed, Klein (251) has suggested that the bacteriostatic and bactericidal effects of oxytetracycline may be independent, involving two different receptors. The bactericidal effect was optimal with rapidly proliferating organisms in small numbers; the bacteriostatic effect was optimal with a large and slowly growing population; and Difco proteose-peptone contained a heat-stable, acid and alkali stable factor which was necessary for bactericidal activity, but not for bacteriostasis.

Pigment formation in *Serratia* was inhibited by chloramphenicol and oxytetracycline and chlortetracycline at levels much below those needed to inhibit growth (252); and the two tetracyclines inhibited growth and temporarily retarded chlorophyll synthesis in lentils, peas, and rapeseed (253).

Paradoxically, chlortetracycline at therapeutic levels was found to inhibit the phagocytosis of *Micrococcus pyogenes* by polymorphonuclear leucocytes (254). The effects of various antibiotics, including the tetracyclines,

on the morphology of various bacterial genera have been carefully studied by Parvis (255) and Pulvertaft (256). Some of the observed changes were considered drug-specific.

#### MISCELLANEOUS

There are only scattered observations bearing on the mode of action of the remaining antibiotics. Phenylalanine or pantothenate caused a striking reversal of erythromycin activity against *Corynebacterium diphtheriae*; and L-carnosine was similarly effective against lower concentrations of the antibiotic (256a). Polymyxin E was found to be bound by *Bacillus subtilis*, *Pseudomonas denitrificans* and *Streptococcus fecalis*, in proportion to their sensitivity to the antibiotic (256b; cf. penicillin page 181). The treated cells released into the medium substances absorbing strongly at 260 m $\mu$ , in amounts proportional to the number of bacteria killed. It was concluded that "the antibiotic combines with and thereby disorganizes structures responsible for the maintenance of osmotic equilibrium within the cell wall." In keeping with the detergentlike structure of polymyxin B, low concentrations were found to inhibit an esterase in *Mycobacterium butyricum* and *M. tuberculosis* (256c).

#### RESISTANCE

##### VARIATIONS IN NATURAL RESISTANCE TO THE ANTIBIOTICS

As was noted in a number of early papers, it is important to distinguish between (a) natural resistance, i.e. the differences in sensitivity between bacterial species or different strains of the same species as they occur in nature, and (b) the resistance developed in originally sensitive strains after their serial transfer through increasing concentrations of the antibiotic. The reason for the widely varying resistance to antibiotics of bacterial strains as they occur in nature has not been clarified.

*Penicillin*.—With penicillin at least three mechanisms are operative. A number of bacteria release penicillinase into the medium. A few species, e.g. *E. coli*, *Proteus morgani*, and *Shigella paradysenteriae* which do not form extracellular penicillinase, are nevertheless able to inactivate large amounts of the antibiotic after it gets into the cell (119); and such rapid intracellular inactivation probably accounts for the resistance. For most bacteria as they occur in nature, however, the primary determinant of resistance to penicillin appears to be the reactivity of as yet unidentified penicillin-vulnerable components of the cell with the antibiotic. In the sensitive cell, these components are highly reactive with the antibiotic, and a relatively low concentration of penicillin in the medium suffices to bring about the lethal degree of combination; in the resistant strains, the penicillin-vulnerable components have a low combining affinity with the antibiotic, and high concentrations of penicillin must be added to the medium in order to effect the lethal degree of combination (117). In a sense, therefore, all bacteria are equally sensitive to penicillin, in that equivalent amounts of bound penicillin produce similar cytotoxic effects, no matter what the "sen-

sitivity" of the organism in terms of the concentration of the antibiotic which must be added to the medium in order to bring about that uniformly lethal degree of binding (120). These considerations do not apply to bacteria which have been made resistant by selective transfer in antibiotic (q.v. page 200)

There is as yet no information as to the degree to which similar considerations may be involved in the varying natural resistance of bacterial strains to other antibiotics. The experimental approach is limited by the availability of isotopically labelled material sufficiently high in specificity.

In contrast to the numerous studies on biochemical differences between resistant variants and the parent sensitive culture (q.v. page 200), there has been but little study of possible biochemical differences between bacterial strains of the same species which differ widely in their natural resistance. Kylin & Lindberg (257) studied coliforms isolated from nonintestinal infections, and attempted to correlate biochemical differences with their varying resistance to a number of chemotherapeutic agents. In general, strains producing acetoin and indole, and utilizing inositol and citrate, were more resistant to streptomycin, chloramphenicol, and oxytetracycline. With *M. pyogenes* an interesting correlation has been found between phage type susceptibility and penicillin resistance (cf. page 205). Barber (276a) has isolated a penicillin-dependent variant of *M. pyogenes*.

*Penicillinase*.—It was shown by Pollock and Pollack & Perret (113, 114) that the fixation of penicillin by *Bacillus cereus* resulted in the formation of penicillinase when the organism was subsequently grown in a penicillin-free medium. The maximum yield following pre-incubation with the antibiotic was obtained when the organisms had been exposed to one unit per ml. At this concentration it was estimated that 80 atoms had been bound per cell. The primary phase of combination was found to be 50 per cent complete in 60 sec. at 35°C., and to involve the specific fixation of penicillin on a cell receptor. Following the combination of the antibiotic with the cell, there was a latent period of 15 min.; its duration was unaffected by cell numbers or by the amount of penicillin which had been fixed. The third and active phase of penicillinase production was inhibited in an anaerobic environment but resumed without lag when oxygen was restored after short anaerobiosis (115). However, if the organisms were kept anaerobic for 100 min., there were profound alterations in their capacity to form penicillinase, as evidenced either by "reversion," a decreased rate of penicillinase production, or by "rebound," a temporarily increased rate of penicillinase production for 15 to 20 min., after which the new rate was established (258). Pollock suggests that the rebound phenomenon may reflect the accumulation of a specific enzyme precursor.

The bound penicillin acts catalytically. In the hour after its addition the enzyme formed may exceed by 20-fold the amount of penicillin added. The enzyme has been estimated to have a molecular weight of 50,000, and to have a turnover number of  $1.3 \times 10^6$  (258a). With both *Bacillus subtilis* and *B. cereus*, Manson, Pollock & Tridgell (258b) found no qualitative difference

between the basal penicillinase and the enzyme formed in response to penicillin. However, the enzymes produced by the two species differed significantly in Michaelis constants, the shape of the pH-activity curves, and immunologic properties.

The induced production of penicillinase by *B. cereus* does not take place at 42°C. (259); but the basal enzyme is unaffected and continues to act at that temperature. With *Micrococcus pyogenes* no adaptive formation of penicillinase could be demonstrated (260). The enzyme was constitutive and largely intracellular, and the rate of its production was reduced by the addition of protein hydrolysate to the medium. In those cultures of *M. pyogenes* which produced penicillinase, the individual organisms were sensitive, and the apparent penicillin resistance of the culture was proportional to the rate of penicillin inactivation (261). Cultures of penicillinase-producing staphylococci were found by Bondi *et al.* to throw off mutants which did not produce the enzyme, and which were penicillin sensitive (262). The high rate of this mutation was further increased by incubation at 45°C.

The changes in resistance brought about in penicillinase-producing staphylococci by subculture in streptomycin or chloramphenicol were found by Barber (263) to be caused in some instances by changes in growth rate, and in others by the selection of penicillin-sensitive variants.

#### DEVELOPMENT OF INCREASED RESISTANCE IN ORIGINALLY SENSITIVE STRAINS

*Adaptation and mutation.*—Most laboratory investigations of resistance have dealt not with the varying resistance observed in nature among species or among different strains of the same species, but rather with the development of resistance in originally sensitive strains by selective propagation in increasing concentrations of antibiotic. The most controversial aspect of this problem has been whether the increased resistance thus obtained represents an adaptive response to the drug, which may then persist for many generations after the drug is removed, or whether instead the antibiotic serves merely as an agent which permits the selective propagation of rare resistant mutant organisms present in the original culture. To the reviewers, the present status of this problem may be summarized as follows:

There is an overwhelming body of evidence that the highly resistant organisms which grow out selectively in the presence of high concentrations of an antibiotic, and which retain their resistance after cultivation for many generations in drug-free media, are in fact initially present in the culture, and do not arise as a result of their exposure to the drug. One may cite the results obtained by Demerec (264), using the fluctuation analysis of Luria & Delbrück; the results of the agar-plate spreading technique of Newcombe (265); the crossing experiments of Lederberg (266) and of Newcombe & Nyholm (267); the results of the replica plate technique of Lederberg & Lederberg (268); the production by Hotchkiss (269) of increased resistance to penicillin by transformation of sensitive *Diplococcus pneumoniae* with DNA derived from resistant strains; the similar production by Alexander

& Leidy of increased resistance to streptomycin in *Hemophilus influenzae* by DNA derived from resistant organisms (270); and the transduction of streptomycin resistance in *Salmonella* by a filtrable agent derived from resistant strains (270a). In the aggregate, these lines of evidence, and others not here cited, indicate apparently incontrovertibly that resistant variants are in fact preformed mutants which grow out selectively in the presence of the antibiotic. The fact that the results of the fluctuation test as applied to the study of antibiotic resistance have been questioned (271) does not materially lessen the cogency of this array of evidence.

The observation that the composition of the medium affects the development of resistance to polymyxin B by *Aerobacter aerogenes* and *Pseudomonas aeruginosa* (272) and to streptomycin by *Hemophilus pertussia* and *Micrococcus pyogenes* (273) can easily be reconciled with the hypothesis that mutation and selection are the origin of resistant variants. Further, the data suggesting that resistant organisms differ in important metabolic activities from the sensitive parent strain (cf. *infra*), and that these differences may develop only after exposure to the antibiotic (274, 275) do not controvert this hypothesis. Even if there were significant and reproducible differences in the metabolic activities of parent sensitive organisms and resistant variants (*vide infra*), and even if it could be conclusively demonstrated that those differences develop only after exposure to the antibiotic, the observation could mean only that resistant mutants differed from the sensitive parent cells in a genetically controlled capacity to adapt (276). The point is nevertheless an important one; and the replica plate technique, which permits the a priori identification of the specific organisms that will give rise to a resistant clone on exposure to the drug, makes it susceptible of experimental study.

From the same culture, and even on a single plate, multiple types of antibiotic-resistant variants may be isolated differing in their resistance, growth rates, morphology, and also in the rate at which they revert toward normal sensitivity (276a, 276b). On the mutation theory, the latter phenomenon would reflect mutation and replacement by the more rapidly growing sensitive mutant. Welsch (276c) has made the observation that in a totally streptomycin-resistant clone of *M. pyogenes*, isolated by a variation of the Lederberg replica plating technique, i.e., without exposure to the antibiotic, individual organisms grown in the presence of streptomycin gave rise to colonies which varied in both resistance and morphology. This observation is pertinent in relation to the claim that resistance develops only after exposure to the antibiotic (cf. *supra*); and Welsch leaves open the question as to whether these differences reflect varying degrees of adaptation, or result from secondary mutations arising during the growth of the colony. The differences between penicillin-resistant strains of *M. pyogenes* as they occur in nature, and as produced by selective propagation in antibiotic may be more apparent than real. Szybalski (276d) has shown that of the penicillin-resistant mutants obtained by selection, approximately 1 in 10,000 is a penicillinase-producer. In contrast, more than 99 per cent of the highly



penicillin-resistant staphylococci isolated from patients produce penicillinase. There is no explanation for the selective advantage obviously possessed by penicillinase-producing mutants in nature.

The area of disagreement with respect to the origin of resistant variants is not the nature of the few highly resistant organisms, but rather the fact that at low concentrations of antibiotics, a large proportion of the bacterial population appears capable of growing out to form resistant clones (276e). Thus, when *Streptococcus fecalis* was inoculated into a poured agar plate containing threshold concentrations of chloramphenicol, and when the paraffin-sealed plate was then incubated, essentially all of the organisms eventually grew out over a period of 50 days to form colonies which were significantly more resistant than colonies of the parent culture (122). As pointed out by Demerec and his co-workers (277) in a somewhat different situation, the difficulty in interpreting such a result lies in the fact that each organism may first have formed a micro-colony of normally sensitive organisms, that a mutation may have occurred in this microclone, and that the resistant mutant has then grown out selectively to form the visible colony. The micro-droplet technique of Lederberg (278) promises a direct approach to the study of this problem.

Evidence in support of the adaptation thesis has recently been submitted by Szybalski (279). Confirming some earlier studies by Akiba (280) and Linz (281), and consistent with the observation (282) that bacteria grown in sub-inhibitory concentrations of streptomycin may become highly resistant, this investigator found that, when washed bacteria were exposed to streptomycin in a non-nutrient medium, the organisms were not killed; and on subculture in a drug-free medium, most of the individual bacteria gave rise to resistant clones. The detailed report of these studies will be awaited with interest.

An observation by Welsch (282a) is, however, highly pertinent in this connection. When resting cells of *Micrococcus pyogenes* and *Pseudomonas aeruginosa* were exposed to streptomycin and the washed cells were then subcultured, there was often a rapid and large increase in resistance. This was, however, interpreted as due to the rapid selective multiplication of pre-existing resistant mutants in the presence of low concentrations of streptomycin which leached out of the treated bacteria, rather than to a mass adaptation of the antibiotic-treated bacteria.

*Mechanism of "acquired" resistance.*—No explanation has been offered for the decreased susceptibility of resistant variants to normally effective concentrations of antibiotic. At least in the case of penicillin, there is reason to believe that the factors responsible are not the same as those which determine the varying resistance of different bacterial species or strains as they occur in nature. In the latter case, resistance has been shown to depend on the fact that penicillin fails to combine with the organisms, either because it is destroyed after it gets into the cell, or, most commonly, because the resistant strain has a low order of reactivity with the antibiotic (119). However, when sensitive bacteria were made resistant by serial passage



through the antibiotic, there was no longer any regular correlation between resistance and binding capacity (117). The reactivity of the resistant variants was in some instances unchanged, in some greater, and in some less; and most of the variants were resistant despite the fact that they had combined with normally lethal quantities of antibiotic. In no instance was the development of penicillin resistance associated with an increased capacity to inactivate the antibiotic (117).

The resistance of *Micrococcus pyogenes* to chloramphenicol (283) and of *Mycobacterium tuberculosis* to streptomycin (284) was similarly not associated with an increased capacity to destroy the antibiotic. However, an unconfirmed report by Mizano (284a), which gives no experimental details, claims that streptomycin-resistant strains do cause a decrease in the residual antibiotic activity of the medium, exceeding that observed with normal controls, and that the factor responsible for the decrease is released by the growth of the organisms.

Merkel & Steers (285) similarly considered the possibility that chloramphenicol reductase activity played a primary role in the development of resistance. Within limits, the more resistant *E. coli* strains became upon serial subculture in increasing amounts of chloramphenicol, the more reductase was present. However, this was apparently not the only mechanism involved. Maximum reductase activity was observed when the strains were relatively slightly resistant, and resistance to chloramphenicol could then be increased to much higher levels without further increase in reductase activity. When highly resistant organisms, capable of growing in 2000  $\mu\text{g.}$  per ml. chloramphenicol, were subcultured, the gradual loss of resistance was paralleled by a loss of reductase activity, until the resistance had stabilized at 10 to 20  $\mu\text{g.}$  per ml. chloramphenicol. At this point, despite the fact that the organisms were more resistant than the parent strain, they possessed less reductase activity. The work of Ramsey & Padron (283) also casts doubt on the determining role of chloramphenicol reductase in the development of resistance to this antibiotic. In contrast to the results obtained by Merkel & Steers with *E. coli*, most resistant variants of *M. pyogenes* reduced chloramphenicol at a lower rate than the parent strain. An investigation of the chloramphenicol reductase activity of the chloramphenicol-dependent strain of *Klebsiella pneumoniae* reported by Gocke & Finland (285a) would be of interest.

Saz and co-workers (286) have recently postulated an altered enzyme protein as an explanation of resistance to chlortetracycline. The organic nitroreductase activity of cell-free extracts of chlortetracycline-sensitive *E. coli* was inhibited by a bacteriostatic concentration of the antibiotic. A similar cell-free nitro reductase derived from a resistant variant was found to be resistant to the antibiotic. No differences could be found in the cofactor requirements of the two reductase preparations.

Resistant variants have been found to differ from the parent sensitive strain in many properties other than antibiotic resistance. These changes are not regular, but vary with the specific organism and antibiotic, and in

most instances there is no evidence that they are causally related to the increased resistance. Thus, a streptomycin-resistant strain of *M. pyogenes* grew luxuriantly in a semi-synthetic medium, while the sensitive parent grew only sparsely; the addition of biotin promoted growth (287). Of 121 tubes inoculated with the parent culture, one which showed abnormally heavy growth was found to contain resistant organisms. This isolation of a resistant variant in a culture not exposed to the drug presaged the replica plating technique of Lederberg, and is highly significant in relation to the question of whether biochemical differences between sensitive and resistant cells are apparent before their exposure (cf. page 201). Chlortetracycline-, penicillin-, and streptomycin-resistant strains of *Streptococcus viridans* were found to resemble *S. fecalis* in their immunologic reactivity (288). McVeigh & Hobdy (289) have described a number of important differences between the metabolic activities of penicillin-resistant *Micrococcus pyogenes* and the parent sensitive strain, and important differences have been noted similarly in chloramphenicol-resistant *M. pyogenes* (290). Beljanski and his co-workers (291, 292) found that strains of *M. pyogenes*, *Salmonella enteritidis*, and *Mycobacterium tuberculosis* resistant to streptomycin accumulated significantly more RNA than the sensitive strains from which they were derived, while their protein contents were unchanged. A strain rendered resistant to penicillin accumulated abnormal amounts not only of RNA, but also of protein, purine, mononucleotides, and acid soluble phosphates (293). Similarly, Smolens & Vogt (273) found that both oxytetracycline- and streptomycin-resistant *Hemophilus pertussis* and *Micrococcus pyogenes* has a higher RNA content than the parent sensitive strain. Their DNA content was unaffected.

Wyss & Sharberger (294) have found an increased amount of a pantothenate-synthesizing enzyme in streptomycin-resistant *E. coli*. Chlortetracycline-resistant *E. coli* fermented salicin more slowly than the parent sensitive strain (295); no differences were found with 13 other carbohydrates. Rosanoff & Sevag (296) found a number of differences in the amino acid requirements and carbohydrate metabolism of streptomycin-sensitive and resistant *E. coli*.

On the other hand, Bondi, Kornblum, & de Saint Phalle (297) could find no important differences between the amino acid requirements of penicillin-sensitive and resistant variants of *M. pyogenes*; Wright, Purcell *et al.* (298) could find no consistent morphological or biochemical variations in strains of *E. coli* resistant to a number of antibiotics; and Espersen (299) found no differences in the growth rates or morphological characters of streptomycin-sensitive and resistant *Mycobacterium tuberculosis*. In a number of bacterial species, variants resistant to chloramphenicol and chlortetracycline were found by Carrère *et al.* (300) to differ from the sensitive parent strains morphologically and antigenically, but not in their virulence or biochemical characteristics. Finally, although the development of resistance to chloramphenicol by *M. pyogenes* was found to be accompanied by a decreased requirement for niacin, thiamin, arginine, and in some cases

for cystine, these altered requirements bore no quantitative relationship to the degree of resistance (284).

An interesting correlation between the phage types of staphylococci and their mutation rate to increased resistance has been noted by Barbour & Edwards (301); and Wallmark (334a) has further observed that the development in increased resistance was associated with a pronounced change in phage-susceptibility.

In keeping with a number of observations with other organisms, Wright, Purcell *et al.* (298) have found that with *E. coli* resistance was produced more slowly if antibiotics were used in combination than if they were added singly. Several workers (302 to 304) have noted that in such cases the use of antibiotics in combination prevented resistance only when both drugs were present at effective concentrations. If the organism was resistant to one of the agents, or if one was used in inadequate concentration, resistance developed at the normal rate. Other reports, however, suggest that, as in the case of bactericidal action (q.v. page 209), antibiotics in combination may synergistically limit or prevent the emergence of resistance even when one of the agents is used at ineffective or only partially effective levels.

A number of observations with respect to the emergence of resistance remain unexplained. It was noted by Saz & Eagle (134) that, when large numbers of organisms were exposed to threshold concentrations of penicillin, the number of resistant variants which grew out decreased rather than increased with increasing density of the bacterial population. Further, when resistant variants were added to such mixtures of large sensitive bacterial populations and threshold concentrations of penicillin, these preformed resistant organisms also failed to grow out. This inhibitory effect of large numbers of sensitive organisms admixed with penicillin on the survival of otherwise resistant variants was species specific, and was not observed with other antibiotics. Independently, Olitzki (305) made a similar observation with *Brucella abortus* and streptomycin. In suspensions of high density, fewer resistant mutants appeared than in suspensions containing smaller numbers of bacteria.

Another puzzling observation, the reverse of that described above, has been made by Pittillo & Foster (306). Strains of *Aerobacter aerogenes* which were resistant to a number of antibiotics (streptomycin, dihydrostreptomycin, chloramphenicol, chlortetracycline, oxytetracycline) were found to release into the medium compounds which inhibited the action of the antibiotic against the parent sensitive strain. The puzzling aspect of these observations was the fact that the reversal of the antibiotic inhibition was specific, not only for the antibiotic, but also for the particular organism.

#### CLINICAL SIGNIFICANCE

The fear that as a result of the widespread use of the antibiotics many bacterial species would develop increased resistance to a degree sufficient to interfere with therapeutic efficacy, has to date proved groundless. For example, with penicillin there has been no authenticated case of a resistant

group A streptococcus, pneumococcus, *Treponema pallidum*, or gonococcus, to name only four organisms against which this antibiotic has been widely used in clinical practice. At present, there are only two clinical situations in which the development of resistance to antibiotics commonly poses a serious therapeutic problem. The first is the case of streptomycin, particularly in the treatment of tuberculosis; and here the problem has been met in part by the administration of the drug only two or three times weekly, and in association with some other agent such as isonicotinic acid hydrazide, or *p*-aminosalicylic acid. Several workers have commented on the fact that therapeutically favorable results may be obtained in the treatment of tuberculosis even when isolates prove resistant to the drug. A plausible explanation of this finding may be that in such cases a large part of the population may remain sensitive (307). Further, a good therapeutic result may not necessarily involve the actual sterilization of the lesion (308, 309). A curious aspect of the development of resistance of *M. tuberculosis* to streptomycin is the fact that, contrasting sharply with the results in man, resistance was observed by Wolinsky & Steeken (310) only rarely in guinea pigs, mice, or rabbits treated for prolonged periods, even in the presence of caseation and pulmonary cavitation.

With the other antibiotics, staphylococci constitute the only clearly documented instance in which an originally antibiotic-sensitive species has become refractory to treatment. No other pathogen has shown comparable changes in sensitivity over the past 5 to 10 years (311, 312, 312a). The clinical difficulty posed by these antibiotic-resistant staphylococci is, however, none the less real for being unique, and is compounded by the fact that many of the strains are resistant to a number of antibiotics (217a, 313). In a recent report, Bondi *et al.* (314) found that of 125 strains studied, 28 per cent were resistant to penicillin alone, 40 per cent were resistant to penicillin, streptomycin, chlortetracycline, and oxytetracycline, 4 per cent were resistant to these as well as chloramphenicol, while only 22 per cent were sensitive by the arbitrary criteria used. One can only speculate as to whether these organisms simultaneously became resistant to a number of antibiotics, or whether, instead, resistance to the various antibiotics has evolved consecutively, as these antibiotics have been used on an increasing scale. Only erythromycin is regularly active against these otherwise resistant staphylococci and, in hospitals in which this antibiotic has been regularly used over a long period of time, an increasing proportion of the organisms isolated are found resistant to it as well (315).

The clinical importance of the antibiotic-resistant staphylococci has been well summarized editorially (316, 317), by Finland & Haight (318), and by Spink (318a). What is the basis of the increased resistance, and why has it developed uniquely with the staphylococci? There seems little question that it is a consequence of antibiotic therapy. In population groups, both in this country and abroad, in which the antibiotics are used only to a limited extent, or not at all, there are relatively few resistant strains (318 to 323). Lepper *et al.*, in one of the few controlled studies, showed that the propor-

tion of strains resistant to erythromycin in a hospital population increased from 0 to 70 per cent when the antibiotic was used alone for a 5-month period, and fell to 33 per cent when penicillin was substituted. Following that substitution, the proportion of penicillin-resistant strains increased from 35 to 83 per cent. Martin & his co-workers (315a) have similarly found an increasing proportion of erythromycin-resistant staphylococci, and on epidemiologic grounds have warned against its unnecessary use. Finland has recently summarized his extensive studies on the changing pattern of resistance to antimicrobial agents (312a).

Although it seems clear that antibiotic-resistant staphylococci have greatly increased in prevalence as a result of antibiotic therapy, it seems equally clear that in many instances, and perhaps most, the increased resistance does not represent either an adaptive change, or a mutational selection within strains which were originally sensitive. Instead, there appears to have been a selective propagation of strains which were resistant to start with, and which over a period of months or years have grown out selectively in a host environment which limited the growth of the normally preponderant sensitive strains. It was recognized soon after penicillin came into general use that many of the staphylococci which were resistant in nature produced an enzyme, penicillinase, which inactivated the antibiotic. Thus, Wallmark (261) has recently found every one of 327 resistant strains isolated from patients to be penicillinase producers. On the other hand, resistant variants derived from sensitive organisms by serial propagation through increasing concentrations of the drug do not produce penicillinase. Since almost all of the resistant strains of staphylococci now encountered in man form penicillinase, it is a natural inference that, in general, these resistant strains have not arisen from strains which were sensitive in 1941 and which have developed resistance as a result of their exposure to the antibiotic, but rather that over the past 10 years there has been a selective propagation of strains which were resistant initially. There are two other observations in support of this view. It was shown by several workers that, when patients carrying penicillin-sensitive staphylococci were admitted to a hospital for treatment and were subsequently found to harbor resistant staphylococci, the latter were often of a different phage type than the original organism isolated, and presumably reflected superinfection or replacement, rather than a change in the original strain (323 to 325b). The findings of Lepper, Jackson & Dowling (326) are significant in this connection. Of 343 pairs of staphylococci isolated concurrently from the nose and throat, 101 pairs were of different phage type, and in most instances varied significantly in sensitivity to penicillin, chlortetracycline, and erythromycin. Similarly, of 831 pairs of organisms isolated from successive cultures, 308 were of different phage type and again varied widely in their sensitivity. These authors also found no evidence that originally sensitive strains became more sensitive under treatment.

One of the most interesting aspects of the increased incidence of antibiotic-resistant strains of staphylococci, and one which strongly supports the

thesis of replacement rather than change as to the underlying mechanism, is the convincing evidence (313, 315, 323, 324, 325, 327 to 330) that the medical and nursing staffs of hospitals and similar centers of antibiotic therapy have served as carriers of these resistant strains, and have thereby largely contributed to their spread in hospital populations.

There is nevertheless reason to believe that, both with penicillin and with erythromycin, increased resistance following treatment may occasionally involve an actual change in the presenting organism. In some cases of apparently monotypic staphylococcal septicemia, organisms of the same phage type were isolated before and after treatment, their resistance having increased many-fold in the course of that treatment (323). The genesis of antibiotic-resistant strains of staphylococci therefore usually appears to be the selective propagation of originally resistant strains, their spread facilitated by the fact that members of the hospital staff serve as vectors. In a few instances, originally sensitive organisms may have become resistant as a result of treatment.

An interesting correlation has been noted by a number of workers between the resistance of staphylococci to a number of antibiotics, and their phage types (324, 325, 330 to 334). The significance of this observation in relation to the mechanism of resistance is not yet clear. An observation by Wallmark (334a) is highly significant in this connection. When penicillin-sensitive strains were made resistant by serial passage through antibiotic, they were then either refractory to the phages which lysed the parent sensitive culture, or much less sensitive. Their phage sensitivity gradually returned to normal on subculture in penicillin-free media.

There has been no final clarification as to why staphylococci uniquely have become antibiotic resistant. Individual strains of staphylococci differ greatly in their penicillin resistance, while strains of, e.g. group A streptococci, or pneumococci are remarkably uniform in this respect. The selective propagation of the resistant strains would therefore have a pronounced effect on the overall resistance of staphylococci, but little effect on the other species (cf. 312a). Not only do individual strains of staphylococci differ in their penicillin-resistance to a greater extent than do most other bacteria, but the intracolonial variation is also much greater in staphylococci (122). In consequence, by selective transfer in increasing concentrations of antibiotic the resistance of staphylococci can be stepped up much more rapidly than that of most other organisms (276).

#### SYNERGISM AND ANTAGONISM

The evaluation of the antibacterial action of antibiotics when used in combination is complicated by the fact that the experimental results often "are discrepant both as between laboratory investigators and clinicians, as well as between investigators. Methods of determination are not identical, nor their interpretation, nor even the significance of the terms" (334b). In assessing this combined effect, one may measure (a) the concentrations necessary to effect a given degree of antibacterial action, (b) the proportion



of organisms which remain alive after an arbitrary period, or (c) the time required to effect a given degree of inhibition. Similarly, if instead of studying a direct effect on a bacterial population, one measures the therapeutic effect in an infection, one may determine either the curative dosages, or the proportion of patients cured. Not only have the parameters of measurement varied, but also the quantitative criteria by which synergism is to be adjudged. Not all investigators have distinguished between (a) a purely additive effect, in which the two drugs together work better than either alone in spite of the fact that they act independently on different members of the bacterial population, and (b) a potentiating action in which one drug augments the action of the other on individual cells. Additive effects alone may contribute materially to the therapeutic outcome. A relatively small dose of agent A may kill, e.g. 99.9 per cent of the bacterial population, but a large multiple of that dose may be required to effect sterilization because of the refractoriness of a small segment of the population to the drug. The same might be true of drug B. Acting together, however, but quite independently, the two drugs might effect sterilization. Clinically a negligible percentage might be cured with a given dosage of either drug alone, but a high percentage when they are used in combination, even when they act independently. Jawetz & Gunnison (335, 336) have given a clear discussion of this point. Unfortunately, the term synergism has been applied both to simple additive effects, and to potentiation phenomena. Thus, two agents have been said to be synergistic if less than 40 per cent of the inhibitory dose of each alone effects inhibition when used together (337). Similarly consistent with an additive rather than a potentiating effect is the criterion that the lethal effect of the drugs in combination be greater than that of the more lethal drug alone (338), or that the percentage of animals protected by the two antibiotics be greater than the sum of the percentages protected by each separately (339). Even the finding (340) that 1/40 of a curative dose of penicillin and 1/7 of a curative dose of bacitracin are together curative in rabbit syphilis is consistent with an independent and additive effect of the two drugs on different members of the microbial population. At first sight, this appears to be a clear-cut example of mutual potentiation; yet each of these fractional curative doses alone kills most of the organisms, and their curative effect in combination could be merely additive.

In other cases, however, the drugs do not act independently, but mutually increase their cytotoxic effects. It has been shown by a number of workers (341 to 347) that the action of penicillin and streptomycin on enterococci falls into this category. Bacteria which are not killed by penicillin alone rapidly become nonviable on the addition of streptomycin, even when the latter is added at a fraction of the effective levels. Conversely, dosages of penicillin which merely delayed growth, combined with sub-sterilizing dosages of streptomycin, were found to effect the sterilization of *E. coli* suspensions (346). Reynolds & Rowley (348) have shown that this synergistic effect of penicillin and streptomycin is not due to an increased uptake of penicillin by the bacteria under the influence of streptomycin. A number



of other antibiotics have been found to be active in combination, even when one of the pair is used in sub-inhibitory concentration (75, 349, 350, 351, 352, 352a, 352b). Similarly, non-inhibitory concentrations of streptomycin were found to promote the action of isonicotinic acid hydrazide on *Mycobacterium tuberculosis*; and the converse was also true (253). Of particular interest is the observation that sub-inhibitory concentrations of a number of antibiotics were effective when used in conjunction with penicillin against a penicillin-resistant *Micrococcus pyogenes* (352a, 352b). These observations, pointing as they do to the fact that antibiotics may mutually affect each other's antibacterial action at concentrations which in themselves do not have an apparent cytotoxic effect, is of obvious central importance in relation to the mechanism of synergism. In some of these cases, however, the criterion of activity was the inhibition of growth in a fluid culture; and the possibility remains that each antibiotic alone at the "sub-inhibitory" concentration was killing the major portion of the population. Under these circumstances the apparent "synergism" could have been an independent and additive effect on different segments of the population.

In the instances in which it has been studied, it has been found that both members of the synergistic pair must be acting simultaneously in order to effect potentiation. The synergistic action is not observed if they are added consecutively, either *in vitro* (354) or *in vivo* (355). The interrelationship between the development of resistance and synergism has been discussed by Klein & Schorr (356) (cf. page 212); and the use of drugs in combination to prevent the emergence of resistance has been discussed in a preceding section.

Pittillo & Foster (76) have made an interesting observation bearing on the mechanism of synergism. Two inhibitors which were reversed by the same metabolite were often synergistic when used in combination, and further, this synergistic effect was reduced by the addition of the reversing metabolite. Thus, the effects of both dihydrostreptomycin and L-tyrosine on *Aerobacter aerogenes* and *Mycobacterium tuberculosis* were reversed by phenylalanine. Similarly, antibiotic 106-7 and chlortetracycline were both reversed by glycine. In both instances, the combinations were synergistic. Two possibilities were suggested in explanation of these effects. The first is "sequential blocking," in which the two inhibitors block the biosynthesis of the same reversing metabolite at different points in the metabolic pathway, and thus potentiate each other. In the second mechanism, which they term "protective inhibition," drug B inhibits the synthesis of a metabolite which would otherwise reverse the action of drug A.

Jawetz & Gunnison (336, 344) have defined synergism and antagonism in terms of rates of action. Synergism was defined as a large increase in the rate of early bactericidal action and the rate of cure of infections, beyond that obtainable by simple additive effects; while antagonism was defined conversely as a decrease in rate of bactericidal action *in vitro*, and a decrease in the rate of cure of infections below that observed with the single more active drug. On this basis, they have divided antibiotics into two groups

with respect to their effects when used in combination. Group I antibiotics, which included penicillin, streptomycin, bacitracin, and neomycin, were frequently synergistic and occasionally indifferent to each other, but never antagonistic. It is to be noted that all are actively bactericidal agents. Group II included a group of agents which are primarily growth inhibitory rather than bactericidal: chlortetracycline, chloramphenicol, and oxytetracycline. These were found to be neither synergistic nor antagonistic with each other. Combinations of antibiotics of Group I with those of Group II could be either synergistic, antagonistic, or neutral, varying with the particular organism, the concentrations used and the duration of their exposure. Group II was subsequently expanded to include erythromycin and carbomycin (357). It is to be noted that these antibiotics also, although bactericidal in high concentrations, are primarily growth inhibitory rather than bactericidal at therapeutically effective levels. It proved difficult to classify polymyxin B (358). Like members of Group I, it was sometimes synergistic with members of Groups I and II; and, unlike other members of Group I, it was not antagonized by any of the Group II antibiotics.

A number of workers have stressed the variability and unpredictability of the effects of antibiotics when used in combination (334a, 335, 336, 359, 359a) and have particularly noted the infrequency of antagonistic effects (349, 350, 360). Although there is no doubt that antagonism can be produced experimentally both *in vitro* and *in vivo* (361 to 363), and although the phenomenon is obviously important in its relation to the mode of action of the antibiotics, its clinical significance is dubious. Ahern, Burnell & Kirby (364) found in an experimental streptococcal infection of mice that chloramphenicol inhibited the bactericidal action of penicillin only if both drugs were given in a single injection. If effective levels of the antibiotic were sustained for a period of days, no antagonism was observed. They concluded that there were "probably few clinical conditions in which the phenomenon is of clinical importance." Jackson, Lepper, Seto & Dowling (365, 365a) found that whether with single or multiple injections, chlortetracycline and penicillin used in the treatment of pneumococcal infection of mice might show either antagonism or addition, depending on the dosages used and the relative times of their administration. The only clearcut evidence of antagonism between antibiotics in their clinical use so far reported is the finding by Lepper & Dowling (366) of an antagonism between penicillin and chlortetracycline in the treatment of pneumococcal meningitis. Ahern & Kirby (367) could demonstrate no antagonism between chlortetracycline and penicillin in the treatment of pneumococcal infections of man; and Walker (368) similarly found no interference between chloramphenicol and penicillin in the treatment of acute streptococcal pharyngitis. Jawetz & Gunnison & their co-workers (335, 335a) have reemphasized the importance of the concentration of the drugs and the duration of their action in determining whether one obtains antagonism, synergism, or a neutral effect when antibiotics are used in combination. Because of the strict time-dose relationships essential for the experimental demonstration of antagonism, its occurrence as the outcome of

clinical antibiotic therapy was held to be most unlikely, if not virtually precluded.

No entirely satisfactory explanation has been offered for the phenomenon of antagonism. Some antibiotics, notably penicillin, erythromycin, and in some instances, streptomycin, are fully effective only on bacteria which are in an environment permitting active metabolism. An antibiotic which inhibits certain metabolic activities might thereby render these organisms relatively refractory to the action of a second antibiotic such as penicillin. Another possible mechanism has been discussed by Jawetz & Gunnison (335).

Kirby & Burnell (369) found that the lysis of bacteria caused by penicillin was delayed by chlortetracycline, oxytetracycline, and chloramphenicol. Lamensans (370) made a similar observation in the case of streptomycin and penicillin. It is difficult to assess the significance of these findings either in relation to the mode of action of the drugs, or the phenomenon of antagonism.

The physician is, of course, primarily concerned as to which combination of antibiotics would be most advantageous in the treatment of infections relatively refractory to any one drug. Unfortunately, the number of possible combinations of two antibiotics is, to say the least, not inconsiderable; and the number of permutations is even greater if one takes the logical next step of evaluating mixtures of three. If one further remembers that the results will vary with the particular organism used, and that the variables of dosage, frequency of treatment, and total duration of treatment materially affect the results obtained, it becomes clear that the evaluation of the efficacy of antibiotics in combination is a truly herculean task. Moreover, as Garrod (371) has pointed out, in the *in vitro* study of antagonism and synergism there appears no present substitute for the actual enumeration of the number of survivors after varying periods of exposure to varying concentrations of drugs, singly and in combination. The value of simplified methods (338, 359, 372) for use as guides to therapy remains to be determined. It remains to be seen, also, whether the recent observations of Klein and Schorr (356) will simplify the approach to the therapeutic problem. Working with drugs at partially effective levels, at which a fraction of the organisms ultimately proved capable of growing out, they have shown, with 11 different organisms and 10 different combinations of antibiotics, that agents to which the organisms rapidly developed resistance regularly showed synergistic effects (by the criteria used); and that the less rapid or the less pronounced the development of resistance to the antibiotics used separately, the less evident were synergistic effects when they were used in combination. If no resistance whatever developed to the agents singly, they were rarely synergistic and often antagonistic when used in combination. As Klein & Schorr have themselves emphasized, it is difficult to assess the relationship of these findings, based on the interaction of antibiotics used at threshold and only partially effective concentrations, to a therapeutic problem which involves the combined effects of drugs when each is used in high concentration, and in which

the emergence of resistance is not usually the factor which determines treatment failure.

## UNTOWARD COMPLICATIONS OF ANTIBIOTIC THERAPY

### TOXICITY

Little has been added to our understanding of the mechanisms of the toxicity of the antibiotics, or of their control. Lane, Kutscher & Segall in 1953 (373) tabulated 255 references dealing with toxic effects of the antibiotics. By 1954 the list, including papers in English only, had increased to 489 (374). Finland & Weinstein (375) have discussed the types of reactions observed, as has Rentchnick (376).

The curious toxicity of penicillin for guinea pigs remains to be explained. The decreased liver phosphomonesterase after an injection of 100,000 units (377) is of dubious significance; no changes were found by Stevens & Gray (378) in the liver glycogen, the non-protein nitrogen, protein and glucose concentrations in the blood, the protein metabolism, and the CO<sub>2</sub> output of the treated animals. There has been an increasing number of reports of fatal anaphylactic reactions caused by penicillin following parenteral administration, and observed most frequently in patients with an allergic history (379, 380). Although several hundred such reactions have now been reported, this number must obviously be weighed against the millions of patients who have been treated. The significance of the demonstration of lupus erythematosus cells in cases of penicillin hypersensitivity remains to be assessed (381, 382). In a hospital survey by the Food and Drug Administration, no serious allergic reactions were found that were clearly attributable to the so-called broad spectrum antibiotics (383).

The present status of the blood dyscrasias caused by chloramphenicol has been reviewed by Welch *et al.* (384) and by Hodgkinson (385). There is as yet no information as to the cause of this serious complication. Prolonged administration of chloramphenicol to normal or irradiated monkeys, or to monkeys previously rendered cytopenic by nutritional deficiencies, did not cause changes in the peripheral blood or bone marrow (386). A major difficulty in the experimental study of this complication is that it is observed in so small a percentage of the patients receiving chloramphenicol, probably less than 1 in 10,000 being affected.

Kuna & Circhie (387) found that guanidine caused convulsions on intracisternal injection, and suggested that this chemical group was responsible for the similar reaction caused by streptomycin. They observed that streptidine had a vestibular toxicity similar to that of streptomycin, but was one-fourth as active on a molar basis.

### SUPERINFECTION

Changes in the host flora resulting from the therapeutic use of the antibiotics, and the general problem of superinfection with antibiotic-resistant organisms have been critically discussed by McCoy (388) in a preceding

volume of this series (cf. also 389). Perhaps the most serious such complication of antibiotic therapy has been the staphylococcal enteritis reported with increasing frequency of recent years, particularly as a complication of treatment with the tetracyclines (317, 318, 390 to 393). The management of this serious and often fatal syndrome is complicated by the fact that many of the strains proved to be resistant to most of the antibiotics in general use. For the present, most of these are susceptible to erythromycin (390, 391, 392, 394, 395), but this promises to be of temporary value only, as the proportion of erythromycin-resistant strains increases (cf. page 206).

Although cases of fungus infection occurring as a complication of antibiotic therapy continue to be reported (e.g. 396), it has been emphasized that the presence of organisms is not necessarily indicative of infection (397, 398). The enhancement of virulence of *Candida albicans* by chlortetracycline observed by Seligmann (399) has now been observed with all three tetracyclines (400). In Seligmann's experiments, inactivated chlortetracycline had no effect. Subsequently, however, derivatives which were devoid of antibacterial activity were found to behave like the active antibiotic (401).

There is evidence (402, 403, 404) that chlortetracycline stimulates the growth of *Candida*; and a similar observation has been made with penicillin (404). However, Sieburth & Roth (405) have found that in chicks and turkey poults infected with *C. albicans* by inoculation into the intestinal tract, the addition of chlor- and oxy-tetracycline to the diet greatly reduced mortality and prevented rather than augmented symptoms referable to moniliasis.

A number of organisms other than *Micrococcus pyogenes* and *Candida albicans* may be involved in superinfection, notably *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Hemophilus influenzae*. An important approach to the mechanism of superinfection is the observation of Bohnhoff, Drake & Miller (406, 407) that previous treatment with streptomycin enormously increased the sensitivity of mice to infection with *Salmonella enteritidis*, the LD<sub>50</sub> dropping from 10<sup>8</sup> to 3 organisms after a single peroral dose of 50 mg. of streptomycin. Significant also is the finding by Jackson & Axelrood (408), consistent with a number of earlier observations, that in experimental infections of mice and embryonated eggs with *C. albicans*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and antibiotic-resistant staphylococci, treatment with chlortetracycline, oxytetracycline, and chloramphenicol may adversely affect the course of infection. They suggest as possible mechanisms (a) effects on the organisms (promotion of growth or enhancement of virulence), (b) impairment of the natural defense mechanisms of the host, and (c) a primary toxicity of the drugs.

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## THE NATURE OF THE PSITTACOSIS- LYMPHOGRANULOMA GROUP OF MICROORGANISMS<sup>1,2</sup>

BY EMILIO WEISS

*Virology Division, Naval Medical Research Institute, National Naval  
Medical Center, Bethesda, Maryland*

The psittacosis-lymphogranuloma (P-L) group of microorganisms has attracted the attention of many investigators, not only because they include important pathogens of man and animals, but also because they appear to have properties which set them aside from other viruses, rickettsiae, and bacteria. As agents of disease they have been discussed extensively [Meyer (1, 2, 3); Coutts (4); Cox (5)] and little, if anything, can be added here to this topic. The primary purpose of this review is to assemble the information which pertains to their characteristics as microorganisms and give an integrated account of the present knowledge of their nature. To achieve this goal, material has been selected for its contribution towards an explanation or clarification of the properties of the viruses, rather than for its intrinsic merit. Thus, many valuable articles dealing with medical, public health, or diagnostic problems have been omitted or cursorily treated. Although the review deals mostly with recent findings, some of the earlier articles have been brought in to insure continuity. Integration of data from different sources has often demanded a ready acceptance of the assumption that findings in one member of the group applies to others. This assumption may in some cases be in error.

Because of the emphasis on experimental work, a narrow definition of the P-L group has been adopted and only those organisms have been included which have been readily grown in chick embryos, and have been classified under the genus *Miyagawanella* [Rake (6)].

### ECOLOGY

Meyer (2) has listed 70 avian species, belonging to 10 orders, as natural reservoirs of Ps (psittacosis virus). They include numerous species of psittacine birds and finches, pigeons and doves, the domestic fowl, duck, and turkey, the fulmar petrel, sea-gulls, and the egret. To this list can be added several mammals which are infected with closely related viruses. They are

<sup>1</sup> The survey of literature pertaining to this review was completed in January, 1955. The author is greatly indebted to Dr. Francis B. Gordon for his interest in the review and his many helpful suggestions.

<sup>2</sup> The following abbreviations have been used in this review: P-L (psittacosis-lymphogranuloma); Ps (psittacosis virus); MP (meningopneumonitis virus); FP (feline pneumonitis virus); Mu (murine pneumonitis virus); Lv (lymphogranuloma venereum virus); EB (elementary body or bodies).

the mouse and hamster, cat, opossum, sheep, and calf (3). Man is the natural reservoir of Lv (lymphogranuloma venereum virus).

In 1951 Meyer & Eddie (7) correctly postulated that any laboratory worker who is willing to carry out a series of autopsies, and inoculate through blind passage in mice various organs of different birds or mammals, may in time discover previously unknown hosts of a psittacosis-like agent. Rubin *et al.* (8) isolated a strain of Ps from American and snowy egrets captured in Southern Louisiana and maintained in a laboratory. The virus, highly virulent for the egret as well as laboratory animals, proved to be indistinguishable from the Borg strain of Ps which was responsible for a human outbreak in the same region in 1943 [Rubin (9)]. Meyer & Eddie (10) isolated a strain from a turkey following outbreaks of the disease among employees of poultry dressing plants in Texas. Contact with turkeys was associated with nearly one-half of the 445 human cases which have occurred during 1954 in the U.S.A. (11). York & Baker (12) isolated from the intestines and feces of calves a new member of the group which produced no disease in the natural host. About 60 per cent of the animals in the Ithaca, N. Y. area appeared to be infected. Isolation of a virus of the P-L group causing abortion in ewes was reported from Scotland [Stamp *et al.* (13)] and good evidence was obtained that the same agent occurs in goats [Meyer (14)]. Another agent, causing pneumonia, was isolated from sheep in California, but its identity as a member of the P-L group has not been conclusively established [McKercher (15, 16)]. The etiological agent of sporadic bovine encephalomyelitis has been recently studied and a close relationship to the P-L group shown [Menges *et al.* (17), Wenner *et al.* (18, 19)]. The agent of cat-scratch disease is another which resembles members of the P-L group, but has not yet been grown in chick embryos or small laboratory animals [Mollaret *et al.* (20)].

The distribution of Ps and Lv is world-wide. Human infection with either virus has been recognized in almost every country having adequate diagnostic facilities.<sup>3</sup> In evaluating the prevalence of psittacosis in various localities from published reports, it should be remembered that human outbreaks which attract the attention of virologists and clinicians are unusual incidents from the point of view of the ecology of the virus, while the natural reservoir of infection in animals may go unnoticed.

The relationship between Ps and its psittacine host was elucidated by Meyer *et al.* (21) and Burnet (22), and was recently discussed by Bedson (23). Survival and wide dissemination in nature are dependent on the ability of the virus to persist in its hosts as a harmless or moderately virulent parasite. The young bird acquires a symptomless infection from its parents while it is still in the nest. A carrier state is set up which can persist for years

<sup>3</sup> Reports of human infection have been very numerous and are not quoted here. For the most part, they were based on complement fixation tests performed on patients' sera, demonstrating rising antibody titers against group specific Lv or Ps antigens. In a few cases, virus strains were isolated.

or even for the rest of the life of the bird. The infection remains symptomless unless something disturbs the balance between host and parasite and allows the latter to gain the upper hand. Captivity and shipment frequently upset the host-parasite relationship and result in outbreaks extending to uninfected birds in the consignment as well as to man. An example of this phenomenon is the marked increase in cases of psittacosis in the U.S.A. and Great Britain beginning in 1952 which followed changes in regulations governing the interstate movement or shipment of psittacine birds (23, 24). It is a valid assumption that the above described host-parasite relationship applies to other avian hosts and to other strains of Ps and ornithosis virus.

Similar conditions probably prevail among the mammalian strains. Murine pneumonitis has been usually recognized in naturally infected mice after exposure to various experimental procedures. Karr's experiments (25) indicate that infection is not acquired by contact, but possibly by cannibalism. York & Baker (12) were able to transmit the bovine virus by feeding and contact. Lymphogranuloma venereum is transmitted primarily through venereal contact. The infection is of a chronic nature and, as the others of the group, may remain asymptomatic (5).

Human psittacosis infection plays no role in the survival of the virus in nature. It can usually be traced to close contact with an infected animal, often a household pet such as a parakeet. Some strains are undoubtedly more virulent than others for man and more likely to infect him. It has been shown that Ps can well establish itself in man, although he is only an incidental host. Meyer & Eddie (26) isolated the virus in a patient 10 years after the original infection and reviewed evidence which indicated that this is not an uncommon occurrence. The latent virus behaved in these cases as in the original host and generally did not cause marked symptoms or infect other persons. Similarly, a latent infection with Ps was established in mice following chemotherapy. Hurst *et al.* (27) isolated the virus from treated mice as long as 277 days after inoculation. Following treatment the mice revealed no signs of the disease and did not transmit it to fresh mice placed in the same cage.

If the assumption is made that viruses of the P-L group stem from one source, it is not difficult to visualize how the spread of infection and differentiation of the virus into strains and species has taken place. The survival of the virus in nature is solidly established by its ability to coexist with its primary host. Transfer to a new host probably occurs under unusual circumstances, but, because of the above illustrated behavior of the virus, this incident often leads to permanent relationships and eventually to the selection of mutant strains. The reports of widespread infections in chickens [Ward *et al.* (28)], turkeys [(10); Irons *et al.* (29)], cattle and other animals [Gerloff & Lackman (30)], which have appeared in the last few years, should alert one to the possibility that new strains and new reservoirs may be found to give rise to fresh economic or public health problems.

## PROPERTIES

*Purification.*—EB (elementary bodies) of the P-L group are readily separated from tissue components, but their instability in an extracellular environment and susceptibility to various physical and chemical agents limit the number of procedures which can be employed for their purification. Infected allantoic fluid, yolk sac, and murine lungs have been found useful starting materials in recent experiments [Crocker (31)]; Zahler & Moulder (32); Gogolak (33)]. Allantoic fluid has the advantage of being relatively free of tissue elements, but the viruses are more rapidly inactivated in it than in other chick embryo fluids [(31); Weiss & Huang (34)]. Purified preparations of MP (meningopneumonitis virus) were obtained from allantoic fluids by two cycles of low and high speed centrifugation [Crocker & Bennett (35)] or by dialysis against distilled water (31).

Highly purified preparations of FP (feline pneumonitis virus) were obtained by Zahler & Moulder (32) from infected yolk sacs by a procedure which included tryptic digestion, adsorption of normal yolk sac components on celite, and cycles of high and low speed centrifugation. Trypsin raised infectivity while slightly, but consistently, reducing the amount of sedimentable nitrogen, possibly by dissolving proteinaceous material surrounding the EB and thus dispersing aggregates [Brown *et al.* (36)]. Celite at pH 7.4 and in concentrations of 0.1 gm. per gm. of yolk sac adsorbed tissue components somewhat more rapidly than EB [Moulder & Weiss (37)]. About 10 per cent of the EB remained in the supernatant in a relatively pure state following trypsin and celite treatment (32). In some of the earlier work (33, 37), tissue components were precipitated with rabbit antisera prepared against normal yolk sac or normal mouse lung, but the instability of FP and Mu in rabbit serum limited the usefulness of this procedure. Sedimentation of FP was accomplished by angle centrifugation at 5000 g for 30 min. (32), of Mu (murine pneumonitis virus) at 3000 g (33). Heavy tissue particles were sedimented in a horizontal centrifuge at 300 g for 10 min. (32). The final preparation represented a 70-fold increase in infectivity per amount of nitrogen over the original trypsinized suspension, and yielded no precipitate with rabbit antiserum prepared against normal yolk sac. Furthermore, when uninoculated suspensions of yolk sac labeled with  $P^{32}$  were added to a virus pool, the reduction in radioactivity at the end of the purification procedure was over 100 times greater than the reduction in virus titer (32).

*Infectivity of purified EB.*—Crocker (31) applied the method of Backus & Williams (38) to the enumeration of the EB of MP. Virus suspensions were mixed with appropriate known concentrations of latex particles, examined under the electron microscope, and the numbers of EB calculated from ratios between EB and latex particles. About 200 to 1000 were required to infect chick embryos by the yolk sac route. An ingenious simplification of the latex particle method was devised by Gogolak (33). The EB of Mu were mixed with known numbers of *Escherichia coli*, the material sprayed onto slides which were then Gram stained and examined under a light microscope.



Observed ratios between the bacterial and viral particles allowed the number of the latter to be estimated. Moulder *et al.* (39) applied Gogolak's method to FP and penicillin resistant FP and found the ratio between EB and chick embryo  $ID_{50}$ s to be approximately 20. The discrepancy between the results of Crocker and Moulder can be attributed to the more efficient detection of EB by the electron microscope and to the greater inactivation of EB produced by Crocker's method. Weiss & Huang (34) found that a linear relationship existed, within limits, between FP concentration and number of cells infected in tissue cultures. This indicated that one particle sufficed to initiate infection. The ratios between EB and  $ID_{50}$ s obtained in the cited experiments thus give the number of EB required for the successful infection by one EB and are, therefore, measures of the physiological state of EB and the degree of resistance of the host cells.

*Physical and chemical characteristics.*—Kurotchkin *et al.* (40) studied the size of several members of the P-L group by electron microscopy. The diameter of the EB images varied from 350 to 500  $m\mu$ , MP being the smallest, Mu, followed by FP, the largest, and various Ps strains and Lv occupying intermediate positions. The bovine strain of York & Baker (12) measured approximately 350  $m\mu$  in diameter. Rake (41) measured over 2000 EB of FP and noted that less than 1 per cent of the particles were considerably larger than the rest, about 750  $m\mu$ ; he identified these particles as initial bodies. Similar observations were made with other viruses [33, 40]; Heinmets & Golub (42); Crocker (43)]. Some discrepancies can be noted in EB measurements reported by the various investigators, but they can readily be attributed to differences in source of the material, purification procedure, or metal used for shadowing. Cosslett *et al.* (44) reported that the EB of Mu obtained from mouse lung measured 293  $m\mu$  in diameter, from rat lung and yolk sac 363 and 411  $m\mu$ , respectively. These differences were more apparent than real, because the heights of the EB, measured from their shadows, were approximately the same.

EB have a tendency to spread on the formvar surface and thus attain a diameter considerably larger than in a liquid suspension. For example, in the studies of Moulder & Weiss (37) FP had a diameter of 423  $m\mu$  and a height, estimated from the length of the shadows and the angle at which the shadowing metal was cast, of 161  $m\mu$ . An EB on formvar is essentially a segment of a sphere with one base, while in a liquid medium it is most likely a sphere. The diameter of a sphere of the same volume as the one measured by Moulder & Weiss can be readily calculated, and is 296  $m\mu$ . The diameter of Mu in solution, obtained from Gogolak's (33) electron microscopic data of 462 and 191  $m\mu$  for diameter and height, respectively, is 339  $m\mu$ . Considering that Mu and FP are slightly larger than Ps, the above diameters are in good agreement with the results obtained by filtration through graded collodion membranes which have shown Ps to measure 200 to 300  $m\mu$  [Levinthal (45); Lazarus *et al.* (46)]. These measurements are also in agreement with the recent studies of Gaylord (47) who examined sections of MP infected chorioal-

lantoic membranes with the electron microscope and found the diameter of EB to be considerably smaller than from smears. The EB of the P-L group are too large for accurate ultracentrifugal studies and only a tentative value of  $10,000 \times 10^{-13}$  can be given for the sedimentation constant of FP (37). If this value is reasonably accurate, and if the density of the virus is comparable to that of other microorganisms of similar size, it would also indicate a diameter in the neighborhood of 300 m $\mu$ .

Although varying considerably in size, the EB of the P-L group are remarkably similar in morphology. They all appear to have a central zone of high electron scattering power with an irregularly convoluted surface and a peripheral zone of lesser density (40). The convoluted surface is obviously an artifact due to the process of drying and can be influenced by the salt concentration of the medium. In distilled water shrinking and folding of the central substance is most marked, but when the virus is dried from isotonic saline and is not washed free of salt, convolution is barely apparent [Moulder (48)]. The outer component in the hydrated particle appears to be a containing membrane with osmotic properties. Even in purified preparations, aggregates of two or more EB and structures somewhat different from the rest are encountered. Some of the structures appear to be developmental forms of the virus.

Viruses of the P-L group have an affinity for basic fuchsin similar to that of the rickettsiae, and retain the dye, after application of dilute citric acid, somewhat longer than tissue elements [Macchiavello's staining method (49)]. However, in contrast to rickettsiae, Mu and FP were shown to have a slight tendency to retain gentian violet [Weiss (50)]. Purified EB of FP withstood decolorization with 95 per cent ethanol for 5 sec., an interval longer than that required to decolorize *E. coli*. But the stain was lost after 20 sec. while *Staphylococcus albus* was not affected (37). Melczer (51) reported that treatment of Lv with primulin induced fluorescence.

The studies by Zahler & Moulder (32), based on phosphorus determinations, indicated that FP has the chemical complexity of rickettsiae and bacteria, rather than of the smaller viruses. Phosphoprotein, alcohol-ether soluble phosphorus compounds, pentose (PNA), and desoxypentose nucleic acids (DNA) were identified. The ratio between PNA and DNA phosphorus was found to be approximately 2.5 to 1, but the total content of nucleic acid appeared to be relatively small. Enzyme systems of carbohydrate metabolism were not demonstrated in purified FP (37), although the conditions of the experiments were very similar to those which detected an independent metabolism of glutamate and pyruvate in typhus rickettsiae [Bovarnick *et al.* (52, 53)].

Crocker [quoted by Moulder (54)] isolated both types of nucleic acids from MP. As early as 1938, Robinow & Bland (55) showed that the EB as well as the larger forms of Ps were Feulgen positive.

**Stability.**—Most of the available information on stability of the P-L group of viruses, summarized by Meyer (1), is concerned with total inactiva-

tion. The kinetics of disintegration have not been studied in detail, although the assumption is often made that inactivation follows the laws of a first order reaction. The complexity of the phenomenon of inactivation is illustrated by some results of Weiss *et al.* (56) which suggested that the infectivity of FP for tissue cultures decreased more rapidly than for chick embryos.

It is apparent that the viruses of the P-L group are among the less stable under ordinary laboratory conditions and in this respect closely resemble rickettsiae. Allen *et al.* (57) showed that at  $-70^{\circ}\text{C}$ . the infectivity of MP suspended in five common diluents was maintained for 11 weeks. At  $-20$  to  $-25^{\circ}\text{C}$ . virus titers declined more or less rapidly depending on the diluent. At  $4^{\circ}\text{C}$ . only the specimens stored in skim milk contained demonstrable virus after 11 weeks of storage. A strain of Ps studied by Bedson & Gostling (58) appeared to be considerably more resistant than the strain used by Allen. Purified suspensions of FP, however, were less stable and a measurable decline was noted after 8 weeks at  $-70^{\circ}\text{C}$ . (37). It was shown, however, that trypsin re-established the titer of old purified suspensions and the decline in titer could be attributed to aggregation of EB rather than loss of infectivity (36). At  $37^{\circ}\text{C}$ . the half life of purified FP was approximately 90 min. (37). The possibility that a cellular environment confers a greater stability on the virus was suggested by some experiments concerned with the fate of the virus in the egg following the death of the embryo and destruction of the host cells [Moulder & Weiss (59)].

The susceptibility of viruses of the P-L group to common physical and chemical inactivating agents appears to be relatively high (1). The pH range of stability of FP was shown to be narrow and its rate of inactivation by ultraviolet irradiation comparable to that of *E. coli* (37, 48). Exposure to ultrasonic waves caused an initial rise in the titer of FP, probably by disaggregating it, but then rapidly reduced it [Hamre (60)]. Ps was relatively stable in concentrations of 25 per cent methanol at  $0^{\circ}\text{C}$ ., but was inactivated by a similar concentration of ethanol [Wagner *et al.* (61)], and ether (1).

*Antigenic composition.*—The antigenic nature of viruses of the P-L group has been elucidated principally by Bedson (62, 63). His contributions, as well as some of the more recent ones by other investigators [Nigg *et al.* (64); Hilleman & Nigg (65); Barwell (66, 67); Sigel & Pollikoff (68, 69)] are presented in simplified form in Table I. Complement fixation tests revealed four main antigens: group specific and strain specific, each associated with EB and with particles smaller than EB. Resistance to heat and to various chemical agents differentiated the stable group specific from the unstable strain specific antigens. On the other hand, as shown in Table I, differences between EB and soluble antigens of the same type were demonstrated with greater difficulty.

The presence of group and strain specific antigens in the same preparations interfered with some of the serological reactions. Complement fixation tests with either human or animal Ps or Lv sera in which fresh homologous or heterologous viral suspensions were used as antigens yielded inconsistent

results. Boiling eliminated strain specificity and converted the virus suspensions into potent antigens, which reacted equally well with homologous and heterologous sera. Strain specific reactions could be demonstrated with fresh viral suspensions and sera from which the group reactivity had been eliminated by absorption. Homologous or heterologous heated viruses were equally satisfactory in removing the group antibody, while preserving the strain specific ones. Similar results were obtained in cross-reaction studies with the agent of enzootic abortion in ewes [Monsur & Barwell (70)].

The group specific antigen was enhanced by other means as well as by heat. Nigg, Hilleman, *et al.* (64, 65) detected a marked increase in activity following treatment of the viral suspensions with phenol, and isolated presumably the same antigen as a potent ether soluble fraction. Further refinements of the technic for the preparation of group specific antigens were reported by Davis (71) and Whitney & Gnesch (72).

Antigenic analysis of the P-L group of viruses was complicated by the fact that a somewhat different antibody response was elicited in mammals than in birds. Hilleman (73) and St. John & Gordon (74) obtained highly specific virus neutralizing antibodies in chickens, but similar responses were not consistently obtained in man or rabbits (23). Often sera from naturally or experimentally infected chickens, and occasionally from other animals, did not fix complement in the presence of antigen, but the presence of antibodies was detected by complement fixation inhibition tests [Karrer *et al.* (75, 76)]. The failure, however, of certain antigen-antibody complexes to fix complement can not be attributed with certainty to the nature of the antigen. It was noted by Schmidt *et al.* (77) that certain human sera stored at  $-50^{\circ}\text{C}$ . exhibited a prozone phenomenon which could be partially eliminated by increasing the amount of antigen in the complement fixation system.

It is not clear to what extent antigens demonstrated by complement fixation tests are the same as those taking part in other reactions. Purified EB were agglutinated by specific antisera and heat-labile and heat-stable antigens were demonstrated [Lazarus & Meyer (78); Labzoffsky (79)]. Viral toxins, which have been associated with EB, elicit antibodies with a high degree of specificity [Manire & Meyer (80)], similar, if not identical, to those neutralizing the virus or responsible for the specific complement fixation reaction. On the other hand, soluble Ps or Lv antigens, treated with dilute HCl, no longer exhibited complement fixing activity, but still produced a skin reaction which, in contrast to the Frei test, appeared to be specific in most of the tested patients (67). Among the group specific antigens belong the hemagglutinins, components smaller than the virus [Hilleman *et al.* (81)]. Although the hemagglutinin activity itself is rapidly lost, the antigenicity is stable as is the case with the other group specific antigens [Gogolak (82, 83)]. Immunity and cross-immunity tests revealed antibody responses which varied with the strain of virus and also differed from the reactions obtained in complement fixation tests. Immunity in mice can be produced by the injection of inactivated vaccine [Bedson (84)] and it appears

that the immunizing potency of a vaccine is related to the concentration of group specific EB antigen [Wagner *et al.* (85)]. But cross-immunity tests carried out with active preparations indicate that some strains elicit a broader spectrum of protection than others and suggest that some type specific antigens are involved in the production of immunity [Beck *et al.* (86); Wagner *et al.* (87)].

The complexity of the antigenic composition of the P-L group of viruses which has become apparent after analysis by various methods can be attributed in part to (a) emphasis on different constituents of an antigenic mosaic or (b) difference in sensitivity in detecting a like complex antigen. EB and soluble antigens of the same type of specificity were shown to be so similar that they can best be interpreted as one rather than two portions of an antigenic mosaic. Furthermore, the presence of both group and strain specific antigens in the EB and supernatant fluids suggests that they are closely associated. Obviously, many of the procedures which reveal group antigens to their best advantage are drastic enough to produce chemical changes and, therefore, give a somewhat distorted view of the antigenic structure of intact virus. It is not unlikely, therefore, as postulated by Fulton (88), that group antigens represent central stable structures and various specific antigens slight modifications of the same molecule. In some cases,

TABLE I  
ANTIGENS OF THE P-L GROUP OF VIRUSES DEMONSTRATED BY COMPLEMENT  
FIXATION TESTS

Antigen	Specificity	Preparation	Destruction by
EB	Group	Virus treated with heat (100 to 115°C.), phenol, formaldehyde, ether, or dilute HCl	KIO <sub>4</sub>
EB	Strain	Active virus or ultraviolet irradiated	Heating above 60°C., storage (except at -70°C.), phenol, formaldehyde, ether, or dilute HCl
Soluble	Group	Supernatant from sedimented virus preparation treated with heat (100 to 115°C.), phenol, formaldehyde, or ether	Dilute HCl
Soluble	Strain	Fresh supernatant from sedimented active virus preparation or ultraviolet irradiated	Heating above 60°C., storage (except at -70°C.), phenol, formaldehyde, ether, or dilute HCl

the group reactions are weakened by the specific surface structures; in others, the specific reactions are obscured by the more powerful group reactions. The presence of a mosaic of closely related antigens is suggested, however, by the occurrence of strains of narrow and broad immunogenic power.

Recently, Moulder (48) noted that the parent strain of FP was not neutralized by a chicken serum prepared against a penicillin resistant mutant. This indicates that specific antigens are subject to genetic variation and accounts for some of the antigenically related but distinct strains which have been obtained.

*Hemagglutinins.*—Hilleman *et al.* (81) noted that allantoic fluid containing high concentrations of MP agglutinated murine erythrocytes. An extensive survey of erythrocytes of other species failed to detect any other cells which were agglutinated by the same virus. The nature of the hemagglutination reaction was studied by Hilleman *et al.* (81) and with another virus, FP, by Gogolak (82).

Hemagglutinins were produced in small amounts by either virus. Titers of 1:64 were relatively high, varied considerably in individual embryos, and were not strictly proportional to infectivity. Optimal activity was demonstrated at a pH near neutrality and in the absence of Ca or Mg ions. Hemagglutinins were sedimented by centrifugal forces of 100,000 g for 75 min., while purified EB, even in high concentrations, failed to display any hemagglutinin activity. However, a similarity between the chemical nature of the hemagglutinins and EB was demonstrated. Rates of inactivation at various temperatures above freezing and of destruction by phenol or formaldehyde were analogous to those of virus inactivation. Ultraviolet irradiation, however, did not affect their activity. In recent experiments Gogolak (83) showed that ultraviolet inactivated EB, free from demonstrable hemagglutinins, elicited the production of antisera which specifically inhibited hemagglutination when injected into chickens. Hemagglutinins appeared to contain at least two substances: one was identified as a lipid, lecithin, the other as a nucleoprotein. Lecithin, as well as a nucleoprotein of identical or closely related serological specificity, were also obtained from ultrasonated purified EB.

There is no evidence suggesting that hemagglutinins are either virus precursors or products of virus degeneration, although these possibilities are not excluded. They can be regarded, on the basis of data presented in a later section, as products of the intense synthetic activity which follows virus infection of the host cells and which results only in small part in the production of active virus.

#### GROWTH

*Cycles of development.*—Bedson & Bland (89) recognized that Ps undergoes a relatively complex cycle of development. Their studies were later extended by them and by other investigators [summarized by Weiss (90)] and found to apply, with minor modifications, to other members of the group.



It was shown that, although EB initiated infection, most of them disappeared rapidly from the infected tissues and nutrient fluids. From 5 to 20 hr. after inoculation, depending on the strain of virus, infection was recognized by the appearance of round bodies, approximately 800 m $\mu$  in diameter, called "initial bodies." There is no doubt that initial bodies multiply by binary fission [Rake & Jones (91)] to give rise to clusters of bodies which become progressively smaller as they become numerous. Eventually they are reduced to the size of EB. The particles are enveloped in a containing membrane and a matrix of greater or lesser density, depending on the strain of virus. With dense matrix the structures appear homogeneous in stained preparations and are called "plaques." Those with a light matrix appear granular and are called "vesicles." Both types of structures develop into large vesicles which may contain plaques as well as granules. About 30 to 54 hr. after the beginning of the cycle, vesicles and plaques break up and release several hundred to a few thousand EB. Virus titers parallel the numbers of EB present and are especially high at the time of their release from vesicles.

Species differences are well-reflected in the morphology of vesicles and length of their cycles. Mu is one of the viruses which was shown to develop most rapidly (about 36 hr.) and to produce clusters and finely-granulated vesicles, rather than plaques. Its vesicles, apparently because of high surface tension, were not readily modified by the environment. FP grew more slowly (48 hr.), rarely produced plaques, and gave rise to vesicles with low surface tension, which apparently extended, as they developed, in the direction of least resistance (90). MP (90) and Ps (89) also required 48 hr. for completion of their cycle and formed large plaques. Lv produced clusters of small plaques (91).

The morphology of the developmental forms of the P-L group is influenced to a certain extent by the environment. The vesicles developed to a considerably larger size and harbored more widely separated particles in the entodermal cells of the yolk sac, especially when grown in tissue cultures, than in smaller unvacuolated epithelial cells or mouse lungs (34, 90). However, some of the strain characteristics of the vesicles were maintained in all tissues, which indicates that the virus, rather than the host, is responsible for the cyclic type of development (90).

Some of the recent experiments have been principally concerned with the obscure phase which follows inoculation and precedes development of the initial body. Sigel *et al.* (92) and Girardi *et al.* (93) determined the fate of MP injected into the allantoic cavity of chick embryos by titrations, at close intervals of time, of the allantoic fluid and of the chorioallantoic membrane maintained *in ovo* or *in vitro*. In the allantoic fluid the virus titer declined at a moderate rate for approximately 20 hr. A logical explanation, that this drop was due to absorption<sup>4</sup> of the virus into the entodermal cells of the chorioal-

<sup>4</sup> The term "absorption" is used in preference to "adsorption" because the experiments which are being discussed do not distinguish between attachment of the virus to host cell wall and penetration.



lantoic membrane, was not tenable because a similar decrease occurred in allantoic fluid maintained *in vitro* under the same conditions in the absence of cells. The decline was attributed primarily to thermal inactivation. It was assumed that absorption occurred but it involved only a small fraction of the virus particles and was not measurable. The titer in the fluid started to increase again 20 hr. after inoculation. The viral titer of the washed membranes, on the other hand, was characterized by (a) a marked increase during the first 4 to 8 hr., which was proportional to the size of the inoculum, (b) a similar decrease followed by a period during which no virus, or negligible amounts, could be recovered, and (c) a very rapid increase starting at the 19th hr. after inoculation.

The initial increase in infectivity was caused by absorption of virus from the allantoic fluid. It could be interrupted at any time by removing the membrane from the egg and placing it in a tissue culture medium free of virus. It became apparent from the experiments of Sigel *et al.* (92) that absorption of the virus to host cells was a slow and inefficient process. In some of his experiments it continued for as long as 8 hr. until all the extracellular virus was inactivated by the temperature of incubation. This inefficiency could not be attributed to a degree of resistance of the entodermal cells of the chorioallantoic membrane, because very similar results were obtained by Weiss *et al.* (56) with FP and entodermal cells of the yolk sac grown in tissue culture. Factors influencing the rate of absorption were not clearly understood and only a few observations were made. In the experiments of Weiss *et al.* (56), procedures which increased the rate of contact between virus and host cells favored absorption. Cultures which had developed well and presented a wide surface to the supernatant were more readily infected than explants. The rate of absorption could also be accelerated by gentle shaking of the cultures. The total amount of virus absorbed could be increased by lowering, within limits, the temperature of incubation. It is possible that the procedure used for the preparation of pools impaired the physiological state of the virus and its ability to attack cells. More efficient absorption was obtained when an active culture in the process of releasing EB was used as the inoculum.

The second step in the infection of the chorioallantoic membrane with MP (92, 93), characterized by a rapid decrease in demonstrable virus, was interpreted as a noninfectious phase of development. That noninfectious virus remained active was evidenced by the fact that membranes taken throughout this period of rapid decline gave rise to similar amounts of virus when grown *in vitro* for an additional 48 hr.

The third step was a very rapid production of infectious virus. *In ovo* the host cells released some MP into the allantoic fluid shortly after its production (92). FP *in vitro*, however, was not released until 36 hr. after inoculation, at a time when the first few vesicles appeared to burst, and massive release occurred only at 48 hr., after the cycle had been completed in most infected cells (56).

The results of Sigel, Girardi *et al.* (92, 93) do not lend themselves to an easy interpretation. It is obvious that the drop in titer which follows chick embryo inoculation with MP is caused primarily by the hazards that the virus encounters in an extracellular environment. But a small portion of this drop unmistakably represents loss of demonstrable infectivity which is not accompanied by total loss of activity and is part of the course of virus development. Sigel & Girardi failed to correlate their results with morphological observations and to reconcile them with the most important finding of other investigators, namely, that viruses of the P-L group multiply by binary fission. Therefore, the implication was made that the cycle of development of MP resembles that of a bacteriophage.

Binary fission in itself does not completely preclude the possibility that viruses of the P-L group multiply by a mechanism similar to that of bacteriophages [see review by Evans (94)] during the pre-initial body phase of development. However, such a possibility must be confirmed by the demonstration that, during that period, the morphological identity as well as infectivity of the virus is lost. Furthermore, the analogy with the virus infected bacterium would be strengthened by the demonstration that the infected host cell is no longer able to multiply and that its reaction, within limits, is independent of the multiplicity of infection.

Bedson & Gostling (58) repeated some of the earlier experiments of Bedson & Bland (89, 95), with Ps injected intraperitoneally into mice, and found little evidence to support the view that the infectivity and the morphological identity of the virus were lost. Although they failed to detect intracellular virus during a brief period immediately following inoculation and prior to the appearance of initial bodies, they attributed this failure, quite reasonably, to the difficulty of recognizing isolated EB in low concentrations in tissue cells. The virus titer remained approximately constant in the spleen of mice until the initial bodies began to divide, at which time it increased correspondingly. Bedson & Gostling (58) offered a partial explanation of the results of Sigel & Girardi (92, 93) by demonstrating that suspensions with a high content of large developmental forms were less virulent than similar preparations containing predominantly EB. Therefore, the reported decline in virus titer could be correlated with already known phases of development.

Weiss *et al.* (34, 56, 90) detected numerous host cells in the yolk sac of chick embryos, as well as in the lungs of mice, which contained two or more separate foci of infection. Furthermore, infection was clearly identified in two cultured entodermal cells of the yolk sac undergoing mitosis, although mitosis was a rare occurrence among them. These observations demonstrate that the reaction in the host cell infected with a member of the P-L group differs in at least two important respects from that of the virus infected *E. coli*.

The development of an EB into an initial body requires further investigation. In the absence of complete information on this stage of development, it appears plausible to assume that a virus which has penetrated a host cell

has temporarily exhausted the supply of a constituent needed for cell invasion. As shown in the next section, viruses of the P-L group are greatly dependent on host enzymes for growth. However, an assumption that their parasitism involves the genes of the host cells, as may be inferred for other virus-host systems, cannot be supported on the basis of present evidence.

*Biochemical aspects of growth.*—Comprehension of growth of a virus requires, among other things, an answer to the following interrelated questions: Is the virus particle biochemically inert, or simply lacking some important enzyme systems? What is the effect of viral infection upon the metabolism of the host cell? What is the nature of the contribution of the host cell to virus growth? In attempts to answer the above questions for the P-L group three lines of investigation have been followed: (a) Efforts have been made to dissociate virus growth from the activities of the host cells; (b) The effects of known or presumptive enzyme inhibitors or activators have been studied; (c) Biochemical events in normal and infected tissues have been compared.

The first line of approach has yielded results which indicate that viruses of the P-L group depend on a high rate of host cell metabolism for growth. They have not been grown in the absence of living cells, despite some elaborate attempts [MacCallum (96)]. In the yolk sac of chick embryos, a close correlation was observed between cessation of FP growth and decrease in oxygen uptake by the host cells (59). Hare & Morgan (97) found that nutritionally deficient tissue cultures did not sustain the growth of the 6BC strain of Ps, but virus growth was restored by the addition of beef embryo extract. This enhancing action could not be attributed entirely to development of a large host cell population, because it was not completely reversed by the mitotic inhibitor colchicine. Stimulation of viral growth was also produced by other reagents such as ultrafiltrate or dialysate of beef embryo extract, autoclaved lactalbumin hydrolysate, or Morgan 199 synthetic medium, which, to a greater or lesser extent, stimulated the activity of the host cells.

The only possible exception to the findings of a close correlation between the virus and its host cell is the observation of Weiss (90, 98) that three members of the P-L group, Mu, FP, and MP, although intracellular in every other tissue, including the bronchiolar epithelium, developed extracellularly in the alveoli of murine lungs. For the most part, vesicles bulged out into the lumina from attachments to various cells or fibers of the walls of the alveoli. This observation was confirmed by Loosli (99, 100) but has not yet been satisfactorily explained.

Recent advances in tissue culture methods [Dulbecco (101)] offer very simple experimental designs for the second line of approach, a study of the effect of specific inhibitors or activators. Ideally, a homogeneous host cell population in immediate contact with the fluid phase should be selected and the study should be limited to the period of a single developmental cycle. Some of the recent tissue culture experiments have not approached this ideal,

and hence causal relationships have remained somewhat uncertain.

The extensive investigations of Morgan (102 to 106) suggest that growth of Ps required pteroylglutamic acid and/or citrovorum factor, adenine, guanine, uracil, and some amino acids in great excess of the amounts needed by the normal host cells. The importance of the first two substances was shown by their ability to reverse noncompetitively the inhibitory action of sodium sulfadiazine. Such a reversal could also be accomplished by *p*-aminobenzoic acid, in amounts proportional to the sulfonamides, but not by purines and pyrimidines. The need for the other compounds was indicated by the action of respective analogues which, in some cases, inhibited virus growth in concentrations which were not toxic for the host cells. The recent reviews by Eaton (107) and Pearson (108) contain many other examples of selective inhibitions.

The third and perhaps most successful line of investigation, comparison of the sequence of biochemical events in normal and infected tissues, has been carried out in combination with the other two methods by Moulder, Zahler, *et al.* (32, 109, 110). They concentrated their attention on a 24-hr. period of greatest FP synthesis, and on the most susceptible tissue, chick embryo yolk sac, maintained *in ovo* or transferred 72 hr. after inoculation to an *in vitro* environment. It was shown that the yolk sac had a somewhat unusual type of energy yielding metabolism. It consumed oxygen for several hours with a mean respiratory quotient of 0.69 in the absence of any added substrate. Respiration was not changed by the addition of glucose, although pyruvate was readily utilized. Oxidation was coupled with the uptake of inorganic phosphate into organic linkage. It appeared, therefore, that normal yolk sac obtained its metabolic energy from high-energy phosphate compounds generated by the oxidation of its large stores of long-chain fatty acids. Viral multiplication was closely associated with the oxidative phosphorylation by the host cells and was prevented by metabolic poisons, such as fluoride, azide, and dinitrophenol, which inhibited the uptake of oxygen or inorganic phosphate. Malonate and fluoracetate only slightly inhibited oxygen uptake and did not interfere with viral growth.

Phosphorus metabolism was studied by injecting  $P^{32}$  into the yolk sacs of infected embryos 24 hr. before their expected deaths and into normal embryos at a corresponding time. Although the distribution of phosphorus among the various fractions was approximately the same in normal and in infected embryos, the rate of DNA turnover was far greater in virus-infected cells and in large excess of the rate needed for virus production. An analysis of purified virus labeled with  $P^{32}$  indicated that the two nucleic acid fractions had 4 to 5 times the specific activity of the protein and lipid fractions, suggesting that, as in the case of bacteriophages, nucleic acids were synthesized at an earlier time than the other constituents.

Biochemical studies have furnished only partial answers to the questions posed at the beginning of this section. It appears that energy for virus synthesis is provided by enzyme systems already present in the normal host

cell. To gauge the relative participation of host and virus in virus synthesis is not possible at this time. Investigations carried out thus far have not detected any biochemical activity by the virus. However, if the assumption is made that the virus simply stimulates its own production by the host cell, it would appear that it is a very inefficient parasite and causes profound changes in its host cell. Synthesis is exaggeratedly stimulated, as in the case of nucleic acids, or carried out through pathways which are relatively alien to the normal host cell and can be selectively eliminated in numerous ways. The susceptibility of the P-L group viruses to the common antibiotics and sulfonamides, discussed in the next section, further suggests that they are not devoid of enzymes. A working hypothesis that they are not biochemically inert is still well-justified.

*Inhibition of growth and selection of mutant strains.*—Members of the P-L group are susceptible, each to a somewhat different degree, to various chemotherapeutic agents active against bacteria. They share with the rickettsiae a susceptibility to the tetracycline compounds and chloramphenicol, but, in contrast to them, they are also susceptible to inhibitors of gram positive organisms such as penicillin, and are likewise adversely affected by sulfonamides and not by *p*-aminobenzoic acid. The chemotherapy of the P-L group has been exhaustively reviewed by Hurst (111). Recently discovered chemotherapeutic agents include several quinoxaline compounds, which, although too toxic for human use, proved to be as effective as the most efficacious antibiotics against Ps and Lv infections of mice (27). Schmidt & von Sprockhoff (112) showed that chlortetracycline was effective in eliminating latent Ps infection in mice. Meyer & Eddie (113) carried out an extensive field trial which demonstrated that the natural Ps infection in flocks of squabs could be eliminated by the administration of chlortetracycline, oxytetracycline, or tetracycline for prolonged periods of time and according to a rigid schedule. Tetracycline proved to be somewhat superior to the other two. Carbomycin, which has a spectrum similar to penicillin, also proved to be highly effective against Ps and Lv, but in contrast to chlortetracycline did not protect mice against intracerebral inoculation [Wong *et al.* (114)]. Erythromycin appeared to have a somewhat similar action [Powell *et al.* (115)].

In addition to their susceptibility to chemotherapeutic agents, one of the chief characteristics of the members of the P-L group which separates them from smaller viruses is their ability to develop resistant mutants. Golub (116) passed the 6BC strain of Ps in inhibitory concentrations of sulfadiazine and obtained partial resistance to the amount used by the sixth and complete resistance by the tenth passage. Resistance to sulfadiazine extended to sulfathiazole and sulfamerazine, and the resistance of the parent strain to sulfanilamide was not altered. After 10 additional rapid passages in the absence of sulfadiazine, Ps proved to be completely resistant to a concentration of the drug far greater than that used for inducing the change in susceptibility. An explanation for the ready production of resistance was given by the

fact that sulfadiazine, although greatly reducing the virulence of the parent strain for chick embryos, did not substantially curtail its ability to multiply. A similar mechanism was probably responsible for the acquisition of resistance to quinoxaline compounds by Ps and Lv through four passages in mice (27).

Moulder *et al.* (39) obtained, by a more prolonged series of passages in chick embryos, a strain of FP which was resistant to penicillin. Maximal resistance was acquired, after one or more intermediate levels, by the 33rd passage in the presence of penicillin. Additional passages, 14 in the presence and 20 in the absence of penicillin, failed further to modify the strain. Resistance appeared to be of a special kind. Small amounts of penicillin produced the same obvious effects on normal and resistant strains, and only when large amounts were used, was a difference noted. It appeared that while the effect of penicillin on the parent strain was proportional to its concentration, its action on the resistant strain became independent of concentration and, within a wide range, consisted of retarding the growth of the virus to approximately one-half its normal rate. Resistance to penicillin was accompanied by increased susceptibility to chloramphenicol, but did not change the effect of chlortetracycline and oxytetracycline. Moulder (54) also attempted to develop a chlortetracycline resistant strain. He succeeded by passing the virus in the presence of chloramphenicol but, surprisingly, the susceptibility to the latter drug was not changed.

Penicillin appears to affect FP in a manner similar to bacteria. Its action is essentially virustatic [Hamre *et al.* (117); Eaton *et al.* (118)] and obviously virucidal only in absurdly large amounts, 240 mg. per ml. (54). It was shown by Weiss (119) that the finely granulated vesicles of FP and Mu, under the influence of penicillin, developed plaques of varying size, morphology, and affinity for dyes. This phenomenon was interpreted as an abnormal growth of protoplasm which was not accompanied by initial body division. Moulder *et al.* (54) described similar changes for the penicillin resistant strain of FP. Crofutt (120) administered penicillin during the vesicular stage of FP development and showed that the virus titer remained approximately the same for at least 36 hr. thereafter. Because of the instability of virus at 37° C., his results indicated that a moderate amount of synthesis of infectious virus had taken place during the process of abnormal plaque formation.

Tissue culture studies have extended and clarified somewhat the above findings (56). Approximately the same number of host cells were infected with the parent and resistant strains of FP in the presence or absence of penicillin. The antibiotic retarded the developmental cycles of both strains to approximately four days. However, only the resistant virus was able to infect additional cells upon completion of the first cycle. Therefore, the principal differences between the two strains appeared to consist in their ability to invade cells and not in their mechanisms of growth. It is interesting to note that enzymes concerned with tissue invasion are the only ones which have been identified in animal viruses [Burnet (121)].



Chlortetracycline, a more effective inhibitor than penicillin of the growth of FP, produced changes of a different nature. Both initial body division and transformation into abnormal plaques were prevented or curtailed [Gogolak & Weiss (122)]. Hurst *et al.* (27) observed that similar results were produced by quinoxaline compounds on Ps. Allen *et al.* (123) showed that chlortetracycline did not interfere with the absorption of MP into the cells of the chorioallantoic membrane or with initial phases of growth, but maintained the virus in a noninfectious state.

The investigation of the mechanism of action of the sulfonamides and antibiotics on the viruses of the P-L group has not yet been vigorously pursued, but it appears to be the most logical approach to the search for independent virus enzymes.

#### PATHOGENESIS AND HOST RESPONSE TO INFECTION

*Tissue and cellular tropisms.*—Members of the P-L group vary considerably in their affinities for different types of tissues and cells. Some of these dissimilarities have been the basis for the specific status given to the viruses in the group [(86); Meyer & Eddie (124)], and for the separation of the agents of trachoma and inclusion conjunctivitis and the P-L group into separate genera [Rake (125)]. In general, the most virulent Ps strains for man were also shown to have the broadest range of host and tissue [Larson & Olson (126)]. They were followed, in order of decreasing breadth of range, by Ps strains isolated from birds, and in turn, by MP, Lv, FP, and Mu (86).

The entodermal cell of the yolk sac is susceptible to all agents of the group. This high susceptibility can be attributed not only to its special kind of metabolism (109), but also to physical and chemical factors. The unusual size of the entodermal cell permits the development of a much larger vesicle than do other tissues (90). The folded structure of the yolk sac increases the proximity of the entodermal cells to each other and favors ready passage of the virus from cell to cell. The yolk itself is probably a favorable medium for survival of the virus (57).

Tissue culture studies revealed no obvious pathological changes in the infected entodermal cell of the yolk sac until the vesicles were fully developed, at which time the host cells lysed. It appeared that destruction of the host cells occurred suddenly, and was possibly due to a disturbance of the equilibrium of the surface tension of the fat globules and that of the virus-laden protoplasm (56).

Members of the P-L group were shown to vary in their ability to grow in cells of the chorioallantoic membrane of chick embryos. The growth of Ps and MP strains was excellent, results with FP were inconsistent, and growth of Mu and Lv was negligible or absent [Williams (127); Francis & Gordon (128)]. This difference possibly represented a variation in stability in allantoic fluid, rather than tissue specificity. Consistent growth of FP in the allantoic cavity was obtained when the inoculum was very large (82) and the initial phase of rapid inactivation of the virus prior to its absorption into



host cells did not reduce it below a critical level. Histological studies indicated that Ps and MP specifically infected epithelial cells of the ectoderm, following inoculation on the chorioallantoic membrane [Burnet & Rountree (129)] and of the entoderm when the inoculum was injected into the allantoic cavity (90). *In vitro*, Ps appeared to have no special affinity for cells of the chick embryo and multiplied in the most readily available cells [Yanamura & Meyer (130)].

Similar differences were found among the various strains in their virulence for mice. They all shared an affinity for the lung, although the virulence of Lv and of some of the newly isolated mammalian viruses appeared to be of a lower order or was acquired only after serial passage (12). Ps, MP, Lv, and the opossum strains were shown to be lethal for mice when injected intracerebrally, while the intraperitoneal route was uniformly lethal only in the case of Ps strains. Only the Borg and egret strains of Ps proved to be virulent for the guinea pig when inoculated by the intraperitoneal route. Virulence for birds was used to differentiate avian from mammalian strains (124). Evidence for the ability of some P-L strains to grow in tumor cells was recently reviewed by Moore (131), who also listed Lv and Ps among the viruses for which no oncolytic activity was demonstrated (132).

It has been shown on many occasions that some of the tissue and cellular tropisms of the P-L viruses can be changed by serial passage under appropriate conditions. The genetic basis of these changes has not yet been investigated.

*Toxin.*—Viruses of P-L group produce toxins which, in part, explain their pathogenicity. This topic, including the recent contributions of Manire & Meyer (80, 133, 134), was carefully reviewed by Cox (135) and need not be treated here. There is no further work to be reported.

*Cellular Response.*—Although the pathology of human and experimental infections with viruses of the P-L group has been described in detail [(1, 4); Fite *et al.* (136)], few observations have been made early in the course of infection which can explain the nature of the pathogenic effect of the microorganisms. It is apparent, however, that violent cellular reactions are elicited by the virulent strains.

Gogolak (137) studied the pathogenesis of Mu infection of mice following aerosol exposure. With highly purified EB no inflammatory reactions could be discerned in the alveoli of the lung during the entire period of development of the virus into vesicles. The first reaction, characterized by an accumulation of heterophils in the alveoli, was apparently initiated by the bursts of the vesicles. It is possible that the vesicles contained a toxin which was responsible for this reaction, although Gogolak attributed it to host cell debris. The early reaction was followed by mobilization of lung macrophages and by the outpouring of phagocytes from the blood vessels into the alveoli. Intense cellular infiltration interfered with vesicle formation and most active virus proliferation appeared to occur at the periphery and not in the center of the inflammatory foci. The infection eventually spread and resulted in

complete consolidation of the lungs and death of the animals by suffocation. Within certain limits a linear relationship was found to exist between time of death of the animals and concentration of the inoculum [Weiss & Segeler (138); Gogolak (139)]. Moulder & Weiss (140) noted that the progress of inflammation following Mu infection was accompanied by a decreased rate of oxygen consumption and an increased rate of anaerobic glycolysis, but a similar effect was obtained in lipid pneumonia induced by the instillation of egg yolk. The above results were attributed to the alveolar structure of the lung which allowed a very large cellular infiltration. Fieldsteel & Preston (141) studied the effect of MP on glycolysis of the brain of the susceptible mouse and the unsusceptible rat. Although some unexplained differences were noted in the metabolism of the rat injected with infected allantoic fluid, none were observed in the mouse.

The above mentioned observations suggest that the mechanism of injury by Mu is a relatively direct one and causes a nonspecific reaction in the host. In fact, under certain circumstances the reaction of the host has been affected without any interference with the rate of growth of the virus, Weiss *et al.* (142) prolonged the life of mice infected with Mu by the injection of radioactive iodine ( $I^{131}$ ) and reduced the survival time by a similar administration of thyroxine. Since virus titers were identical in normal and thyroxine-treated mice and thyroxine was effective in reducing survival only when given early in the course of infection, the effects could most logically be attributed to a change in the cellular response of the host. Groupé *et al.* (143) have shown that xerosin suppressed pneumonia caused by Mu and other pneumotropic viruses, although it did not reduce the growth of the respective agents. The most plausible explanation of this action is a reversal of the heightened tissue permeability induced by inflammation. Some of the results of Groupé suggest that, in the absence of inflammation, the virus multiplies to a somewhat higher titer than under normal conditions.

The complex pathological changes produced by Lv (4) indicate that the pathogenic effect of this virus is considerably more complex than the one described above for Mu.

**Immunization.**—The persistence of agents of the P-L group in their hosts in a state of well-adjusted parasitism has already been discussed. This characteristic of the infection of the P-L group suggests that the immune reaction of the host consists of the production of limited barriers to the spread of the viruses. It is explained, in part, by the findings of Bedson (84) who showed that multiplication of Ps was not completely prevented in mice hyperimmunized with formalinized vaccines. Wagner *et al.* (85) showed that immunity was of a high order when both immunizing and challenging doses of Ps were given by the intraperitoneal route, but mice hyperimmunized by the intraperitoneal route with killed vaccines resisted, at best, 10 intracerebral or intranasal LD<sub>50</sub>s.

A more effective response was obtained with living vaccines. Wagner & Victor (144) inoculated guinea pigs intradermally with various virulent

strains of Ps and MP and showed that resistance was developed to intratracheal challenge with 40 respiratory LD<sub>50</sub>s, although no pulmonary lesions were induced. These results are in agreement with the earlier experiments of Rivers & Schwentker (145), who immunized monkeys against intratracheal inoculation by intramuscular injection of virulent Ps.

#### TAXONOMY

*Taxonomic problems within the group.*—Meyer & Eddie (124) have recently discussed the classification of agents within the P-L group, and have critically reviewed the methods, largely based on immunological differences and virulence for various hosts, which have been used for the differentiation of strains. However, the problem of distinguishing between strain and species has not been satisfactorily solved. The primary host of the virus has been used most commonly to separate species and, in some cases as, for example, the mammalian viruses, this appears to be entirely justified. Avian strains have been sometimes separated into "psittacosis" and "ornithosis" viruses [Meyer *et al.* (146)], the first designation applying to the psittacine strains, the second to those originating from pigeons and other non-psittacine birds. But this separation can not always be clearly made [Coles (147)]. Several strains, such as MP, San Francisco, and Illinois have been isolated only from an incidental host, i.e. man, and their origin and proper classification remain a matter of doubt (125). The generic name *Miyagawanella* (6, 125) has been widely accepted for the P-L group. However, Meyer (148) pointed out that the work of Bedson *et al.* (149) on the etiology of Ps antedated that of Miyagawa *et al.* (150) on Lv, and proposed the name *Bedsonia* for the psittacosis group.

*Related microorganisms.*—The agents of trachoma and inclusion conjunctivitis have generally been included in the P-L group with some reservation. They resemble Ps and Lv in morphology, tinctorial properties, cyclic development, and susceptibility to sulfonamides and antibiotics. But because of the host range, which is restricted to man and a few primates, detailed comparisons between the two groups of organisms have not been possible. During the past few years exhaustive, but not entirely successful, efforts have been made to grow the agents of trachoma and inclusion conjunctivitis in laboratory animals or chick embryos and to study their basic properties.

Ito *et al.* (151) detected particles varying in size from 60 to 250 m $\mu$  in electron microscopic studies of purified human trachomatous material. The particles had some resemblance to EB of Ps, but the envelope typical of the P-L group was seen only in the larger particles immediately after purification and was readily lost upon storage. The findings of Rake *et al.* (152), which had suggested a serological similarity between the P-L group and trachoma, have not been confirmed in recent investigations (3). Some of the attempts to grow trachoma or inclusion conjunctivitis in mice or chick embryos have yielded very encouraging results, but unequivocal proof that they have achieved their goal has not yet been obtained [Arakawa *et al.*

(153, 154, 155); Poleff (156)]. The extensive investigation which is now being carried out by a large group of Japanese workers [Kirisawa (157)] on trachoma will undoubtedly contribute to the understanding of the nature and proper classification of its etiological agent. But at the present time the exact relationship to the P-L group is still obscure.

*The position of the P-L group among microorganisms.*—In the sixth edition of *Bergey's Manual of Determinative Bacteriology*, Rake (6) placed the P-L group, together with the rickettsiae and bartonellae, in the order *Rickettsiales*. The family *Chlamydozoaceae* includes, in addition to the P-L group or *Miyagawanella*, the trachoma group, or *Chlamydozoon*, and the coccoid agents responsible for conjunctivitis in cattle and poultry, or *Colesiota*. Meyer (148) pointed out some of the differences between the agents of the P-L group and rickettsiae and expressed the view that their inclusion in the same order was not justified. Similar opinions were expressed by other investigators [Perrin (158); von Sprockhoff, (159)].

The most fruitful working hypothesis of the nature of the P-L group has been that they are neither typical rickettsiae nor typical viruses, but lie somewhere between these two groups of organisms. Whether they should be grouped with one or the other depends not only on our judgment of their nature, but also of the nature of rickettsiae and viruses. Recent advances in the field of bacteriophagy have so completely changed concepts of host-viral relationship (94) that they have placed a new heavy responsibility on animal virologists to prove or disprove that the new findings apply to animal viruses. Hypotheses based on superficial similarities and analogies with bacteriophages have proven time and again to be misleading and can not be accepted. They must be based on experiments as painstaking as those carried out with the latter group of organisms. In the case of the P-L group, a start in that direction has been made.

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# PLAGUE<sup>1</sup>

BY GEORGES GIRARD

*Chef du Service de la Peste, Institut Pasteur, Paris, France*

Plague continues to be the subject of investigations which, in the course of the last two decades, have led to some important findings. These concern: the etiological agent, immunology, epidemiology, prophylaxis, and therapeutics. We have made a critical presentation of the most outstanding studies in the order given above and will conclude with a discussion of the plague problem as it exists throughout the world at the present time.

## THE ETIOLOGICAL AGENT

*Varieties of Pasteurella pestis.*—The causative agent of plague was for a long time considered as a homogeneous species. Present-day investigations have led to the notion that there are three biological varieties of *P. pestis*, each of which is found in a particular part of the world. This distribution is important in regard to epidemiology. Pollitzer (1) undertook the task of assembling all the documentation relative to the differences in the biochemical behavior of *P. pestis*, and devoted a long discussion to this subject in his recent monograph. Berlin and Borzenkov first distinguished two types of *P. pestis*: one, continental, glycerol-positive; the other, oceanic, glycerol-negative. The continental type seems to have been the causative agent in the ancient pestilence centers of Southeast Russia, Central Asia, Mongolia, Manchuria, the Transbaikal area, and Central Africa, in which the organism was perpetuated through wild rodents. The oceanic type provoked the modern pandemic which, originating in Yunnan in 1894, spread over the seas into the five continents, the rat and its fleas having been the original and subsequent agents of transmission. This is the only type known in Europe, the United States, South America, Ceylon, Egypt, India, Indo-China, Java, North Africa, Senegal, Sumatra, Thailand, and Madagascar. The continental type of *P. pestis* is itself divided into two sub-types according to whether or not the culture reduces nitrates to nitrites. Organisms which possess this reducing ability will produce nitrites from proteinaceous material when cultured in broth which does not contain nitrates. This nitrite-positive variety is found south of the Caspian Sea, in the lower Volga region, in Iranian Kurdistan, Turkey, and Iraq; but the limits of the area in which it is distributed remain to be defined. Devignat (2), on the basis of historical considerations, has proposed the following classification, which, utilizing a trinomial denomination, has the merit of agreeing with the rules of nomenclature:

<sup>1</sup> The survey of the literature pertaining to this review was concluded in May, 1954.

*Pasteurella pestis*

var. <i>orientalis</i> .....	Glycerol 0, nitrites +
var. <i>antiqua</i> .....	Glycerol +, nitrites +
var. <i>mediaevalis</i> .....	Glycerol +, nitrites 0

A reservation is made as to the constancy of characteristics which have led to the introduction of the variety *mediaevalis* in the continental type. It is incontestable that the stability of behavior of a strain of *P. pestis* with regard to glycerol is recognized by all the authors. We have never observed a glycerol+strain become -, or vice-versa, regardless of its origin, or of the persistence or disappearance of its pathogenicity.

All the investigations with which we are here concerned have employed the oceanic type (= var. *orientalis*) of *P. pestis*. But there is every reason to believe that this information is valid for the three varieties which differ only in the biochemical behaviors mentioned above.

*Morphological characteristics. Capsule or "envelope?"*—It was recognized after the work of Rowland that *P. pestis* was surrounded by an "envelope," particularly marked in those cells cultured at 37°C. According to Sokhey (3), a true capsule can be demonstrated surrounding all plague bacilli, whether cultured at 28° or at 37°, if one employs the staining technique of Churchmann and Emelianoff. Amies (4) contends that "the so-called envelope of *P. pestis* is nothing more than a particularly well-developed bacterial capsule." The arguments invoked by Amies deserve to be taken into consideration as we will see when discussing immunology.

According to Wei *et al.* (5), the bipolar staining of *P. pestis* is due to the presence of chromatic bodies, changes in which during cellular division suggest a nuclear system.

*Growth and nutritive requirements.*—The growth of *P. pestis* is always slow. Sokhey (6) has shown that the lag phase is appreciably reduced if the inoculation is made into a filtrate of a culture of *P. pestis* itself. According to this author, synthetic media are lacking in certain substances which appear only after the breakdown of part of the organisms in the inoculum. In the filtrate from a 6-hr. culture these essential substances are available. Girard & Gallut (7) have confirmed this finding. They observed that growth in nutritive broth did not occur when light inocula containing only a few hundred bacteria were employed, whereas growth was rapid and flourishing if the filtrate of a several-day-old culture of *P. pestis* was added to this same broth. But according to Girard (8), broth may contain some inhibitors, because by substituting for it solutions of certain peptones, he has always obtained more rapid growth than in the case of a mixture of broth and the peptone solutions. In this way, Tchan (9) succeeded in cultivating *P. pestis* starting from a single cell isolated by means of a micromanipulator. He stated in that case that the average time required for cellular division was about 4 hr. This relatively long division time enables one to understand why the phase of logarithmic growth of *P. pestis* is retarded compared to that of most pathogenic microorganisms.

Several authors have tried to culture *P. pestis* in synthetic media. Proline, phenylalanine, and cystine are essential according to Rao (10). But Hill & Spurr (11) found that the nutritive requirements vary according to the incubation temperature. Rockenmacher *et al.* (12) advocated a chemically defined medium, SC (synthetic-casein), which contains the amino acids of casein in proportions identical to those in native casein. Casein hydrolysate media are being employed with increasing frequency in the preparation of cultures for use in studies of metabolism and of antigens of *P. pestis* [Englesberg & Levy (12<sup>a</sup>)].

*Dissociation.*—In general, the problem of the dissociation of *P. pestis* has been poorly posed and this has led to equivocal interpretations. There has been an attempt to find a correlation in the appearance of the colonies analogous to that of the S and R types of *Salmonella*, and it is this which has caused the confusion. In fact, *P. pestis* always corresponds to the R type if one considers its antigenic constitution (see below). As Pollitzer (13) has aptly expressed it in summarizing the viewpoints of authors who have studied the problem of dissociation of *P. pestis*, the behavior of this microbe is strictly individualistic.

*Factors influencing pathogenicity. Virulence and toxicity.*—Virulence, considered in this review essentially as the ability of a microorganism to multiply abundantly in an animal and to produce lesions, and toxicity are the two properties of *P. pestis* which interact to determine its pathogenicity, this latter term being used as a synonym for virulence by many authors. But strains considered as avirulent on the basis of the above definition can be pathogenic if they are sufficiently toxic. Is it possible, *in vitro*, to differentiate between a virulent and an avirulent strain? Some investigators have studied this problem, but there is no good foundation for an *in vitro* distinction between such strains. Rockenmacher (14) believes that there is a relationship between catalase activity and virulence of *P. pestis*. Actually, it is only *in vivo* that the distinction becomes apparent. According to Englesberg *et al.* (15) a strain is virulent if it causes death from septicemia in a mouse which has received a subcutaneous injection of not more than 300 germs. A strain is said to be avirulent if 10,000 or more germs are tolerated by a mouse. This distinction is arbitrary because it does not take into account intermediate strains, said to be of attenuated virulence. The rate of multiplication of these strains in an organism is slow, but real, if one inoculates relatively high doses, but they do not cause any apparent damage in the mouse or any other susceptible rodent. This is the case, for example, with the EV or Tjiwidej strains employed as living vaccines. We must call attention again to the interesting claims of Otten (16) who showed that, from a virulent strain, one could isolate some colonies entirely devoid of virulence. This change in pathogenicity is not necessarily accompanied by changes in the appearance of the colonies on agar. It is likely that this diversity in the virulence of cells of the same strain is responsible for the diminished and sometimes totally lost pathogenicity of certain subcultures of strains of

*P. pestis* rather soon after their isolation from human or animal organisms.

*Conservation of viability and virulence of P. pestis.*—Among the non-sporulating pathogenic microorganisms *P. pestis* is perhaps the one that retains its viability for the longest time in the laboratory. It is essential that the cultures be kept at a low temperature (about +5°C.), be protected from light, and that the condensed moisture necessary to maintain a certain degree of humidity is not allowed to evaporate when the agar cultures are not in sealed tubes. Francis (17) has successfully transplanted 25-year-old cultures; they still retained a certain degree of virulence. Sokhey recommends desiccation after freezing [cited by Pollitzer (18)]. Passage through susceptible animals is the most successful method of increasing the pathogenicity of strains whose virulence has decreased. Blanc & Baltazard (19) have shown that the organisms remain virulent for 16 months in cadavers of the flea (*Xenopsylla cheopis*), kept in sealed tubes in the ice box.

*Metabolism of P. pestis.*—Our information on this point is still very fragmentary. Among recent investigations we might mention those of Levine *et al.* (20) on the breakdown of serine, and those of Santer & Aji (21) on the terminal oxidation processes by non-proliferating cells and extracts of *P. pestis*. This latter study is interesting as it may shed some light upon the pathology of plague, that is on the mechanism of formation of certain visceral lesions, notably of the liver which is always severely affected in the toxemia of plague (personal communication from K. F. Meyer).

*Bacteriophage action.*—The plague bacillus is highly susceptible to the action of bacteriophages and this property is common to all strains of the three varieties of *P. pestis*. There has never yet been a strain isolated from man or animal which was resistant to lysis. This susceptibility persists throughout subcultures regardless of the date of isolation or degree of virulence. We possess phages which exert their activity at a dilution of  $10^{-9}$ . Are those phages specific for *P. pestis*? One would be inclined to answer in the affirmative when the lytic agents have been obtained from the intestinal contents of plague-infected animals [Couvry (22)], or from rats or their fleas during an epizootic as noted by Girard (23, 24) in Madagascar. However, Hauduroy & Ghalil (25) isolated lytic agents, which act upon *P. pestis*, from material not in any way associated with manifestations of plague. Flu & Flu (26) have made similar findings with canal waters in Holland. Furthermore, coli-phages can lyse *P. pestis*; reciprocally, phages of the plague bacillus are capable of lysing *Salmonella* and *Shigella*, species [Lazarus & Gunnison (27)]. A still more striking illustration of lack of specificity is illustrated by the work of Girard (28) in which he was able to regenerate a dysentery phage, which had almost entirely lost its activity for *S. dysenteriae*, by transplanting it to a culture of *P. pestis* which it immediately lysed. The dysentery bacillus was susceptible to the action of this phage passed on *P. pestis*.

Among the pathogenic microbes, no other species exhibits such homogeneity in its susceptibility to lysis by phage except *P. pseudotuberculosis*

which is linked to *P. pestis* by certain common characteristics, and particularly by its behavior with respect to the same phages (28).

The secondary cultures (mutants) which appear after various intervals, but not regularly, in lysates of *P. pestis* kept at laboratory temperature always differ from the original type, often morphologically, but especially in a reduction of their virulence (29). Girard (30) believes that the existence of these mutants of *P. pestis* suggests the possibility of a reversibility and that, considered from this viewpoint, the question indisputably presents interesting considerations in relation to epidemiology. He has emphasized this with regard to certain laboratory findings which argue in favor of this reversibility.

#### IMMUNOLOGY

The study of the immunological processes that enter into play in plague infection has recently prompted considerable work which has enriched our knowledge of this subject, although the question is far from being exhausted. It is fitting to pay a special tribute to the workers of the George Williams Hooper Foundation in San Francisco who, under the direction of K. F. Meyer, have made a rich contribution to this research, but we should not fail to recognize earlier contributions which laid a foundation for these present-day investigations.

An essential, distinguishing characteristic of *P. pestis* is that its antigens are devoid of lipids. After Pirowsky [cited by Levy-Bruhl (31)] had shown that *P. pseudotuberculosis* and the EV strain of *P. pestis* had no "complete" antigen (according to the definition of Boivin, a polysaccharide-lipid-protein complex), Girard (32) confirmed this finding with virulent *P. pestis*. These two organisms then differ in this regard from other pathogenic species, first of all from *Pasteurella* in the strict sense and from *Salmonella*, or *Brucella*, in which the "complete" antigen represents the endotoxin. Consequently, we will devote some attention to the toxin of *P. pestis*, also considered an endotoxin by early workers such as Besredka and Rowland, before passing on to the study of antigenic components.

*The toxin of plague.*—Toxic phenomena occupy an important position in the symptomatology of plague, a factor which was noted by the early workers and masterfully described by Simpson (33) in 1905. But, in the light of studies on microbial toxins, the toxin which is found in filtrates of broth cultures or in extracts of *P. pestis* cultured on agar has the characteristics of an exotoxin; being proteinaceous in its chemical composition and neurotropic in its physiological effects. This toxin is thermolabile. The combined action of formaldehyde and a temperature of 38°C. transforms it into an anatoxin which is antigenic, and partially protective for the rat and mouse. But Girard & Sandor (34) and Sandor *et al.* (35) have reported that, contrary to the case with true exotoxins, agglutination, precipitation, and protective activity induced by sera of horses which have been intravenously hyperimmunized with the anatoxin or with the total microorganisms, were

elicited for the most part by euglobulins and only in a small part by pseudoglobulins. This is contrary to observations with diphtheria and tetanus toxins. Thus, in the case of *P. pestis* an entity which is proteinaceous in nature and is attached to the microbe, brings about the formation of antibodies of an antibacterial type, whether it be injected intact or in solution. There is then a profound difference between the true exotoxins (as the diphtheria toxin), true endotoxins (typhoid toxin), and the plague toxin which occupies an intermediate position.

*Antigenic structure of P. pestis.*—There are two names which should be remembered among the pioneers who opened the way for subsequent investigations: a) Rowland (36) who in 1910 expressed the opinion that *P. pestis* contained two distinct proteins, one soluble in water and containing the immunizing and toxic substances, the other insoluble, neither toxic nor antigenic; and b) Schütze (37) who in 1932, using serological methods, confirmed the existence of two antigens: one the "envelope," present especially in cultures grown at 37°C. and the other somatic, constant at all temperatures. These points of view as a whole have been confirmed by authors who have re-examined these studies, employing new techniques and have specified the nature of the antigens whose presence was revealed by Rowland and Schütze and later by Bhatnagar (38).

Using an appropriate technique, Baker *et al.* (39) have characterized three antigenic fractions of *P. pestis*. Fraction 1 is non-toxic and is composed of antigen 1A, a protein associated with a polysaccharide, and an antigen 1B, a protein without a polysaccharide. These two substances have some common properties. Injected into the rabbit, they elicit the formation of agglutinins against *P. pestis*; the serum protects the mouse against plague experimentally but does not neutralize the toxin. These antigens are effective in immunizing the mouse but not the guinea pig. Fraction 1 is found especially in 37°C. cultures of virulent bacteria and of certain avirulent strains which are immunogenic. It corresponds to Schütze's "envelope" antigen. Antigen fraction II is toxic for rats and mice, and the antiserum obtained from the rabbit neutralizes the toxin, but agglutinates *P. pestis* only slightly, if at all. Fractions I and II are water-soluble. The third fraction is an insoluble residue consisting of proteins which contain an antigen protective for the guinea pig.

Seal (40) has extracted five protein fractions from cultures of *P. pestis*. One fraction, A, is highly antigenic and represents the "envelope" of Schütze; the other four constitute antigen B, which is somatic and common to all strains of *P. pestis* and to the pseudotubercular organism, while A is present only in protective strains of *P. pestis* and absent from all nonprotective strains and from *P. pseudotuberculosis*.

The investigations of Amies (4) make an important contribution to this study and appear to give a definitive answer to the question: what is the significance of the "envelope?" We saw above that this author considered it as a true capsule. Employing an original technique, he examined drops of



fresh preparations of *P. pestis* cultures mixed with India ink (as did Rowland in 1912) and confirmed the authenticity of the capsule. He observed that on exposure to thiocyanate (optimum concentration 0.5 N), the capsule swells, and then dissolves and disappears leaving the microbial cell completely exposed. The extraction is carried out at a temperature of 37 to 45°C. and is arrested when dark field observations indicate it has gone to completion. The capsular solution has a high level of immunizing and precipitating properties. The capsule represents 2 per cent of the dry weight of the microorganism and is composed of proteins associated with a small amount of hydrocarbon. A few micrograms of this purified antigen are sufficient to produce a strong immunity in *Mastomys natalensis*. Amies notes in conclusion that the antigen is resistant to boiling for 15 min. He believes that it corresponds to fraction 1A of Baker *et al.*

The significance of the work of Amies deserves to be emphasized. Thanks to the convenience of observing preparations mixed with India ink, it is possible to choose those strains which are most suitable for the production and extraction of the capsular antigen. Does there exist in the case of *P. pestis* an antigen analogous to the Vi antigen of *Salmonella*? The Russian authors, Fadeeva, Gheltenkoff, and Korobkova, cited by Pollitzer (41), believe that one does exist, but Jawetz & Meyer (42) do not. There has not yet been enough investigation on this subject to give a conclusive answer. The subject deserves to be reinvestigated in the light of studies by Felix *et al.* (43) and Jude & Nicolle (44) which show that the production of the Vi antigen by *S. typhi* is a function of the culture incubation temperature. Furthermore, does not the substance of the envelope take the place of the physiological Vi antigen, for we have seen that it is this which protects the microbe against phagocytosis?

In truth, the incontestable progress of immunology does not always enable one to distinguish virulent from avirulent *P. pestis* bacilli by means of serological tests. Both contain varying amounts of the same antigens which in the rabbit produce antiserums possessing identical properties. The most important contribution of this work is the possibility of immunizing sensitive animals (except the guinea pig) and man with a "chemical" atoxic vaccine, if one can thus qualify the antigens of Baker *et al.*, of Seal, and of Amies. Meyer *et al.* have assembled a substantial documentation on this subject which calls for some comment.

*Anti-plague vaccines and fraction IB of Baker et al.*—For Meyer *et al.* (45) the value of an anti-plague vaccine, living or not, is a function of its fraction IB content. A highly purified fraction immunizes mice, rats, and monkeys, but not guinea pigs. Most authors consider that man's behavior with respect to plague resembles that of mice rather than of guinea pigs. This opinion is at least partially justified by the fact that man, like rats and mice, is sensitive to the toxin while guinea pigs are not; but let us note well that we are dealing with a toxin prepared *in vitro*. In this respect, the guinea pig represents the true test of the virulence of *P. pestis*. Under these conditions, the

residual antigen which remains in the insoluble fractions and which as we have seen protects the guinea pig would not be indispensable for human immunization. This opinion is perhaps too limited. But is one authorized in comparing a living with a killed vaccine to take into account only their fraction IB content? It has long been known as the result of experimentation that a living avirulent strain is not necessarily capable of producing immunity. Girard emphasized this in 1937 (46) in response to a communication of Sokhey & Maurice (47) who had contrasted the efficacy of heated vaccines with the mediocre activity of certain living vaccines prepared in Bombay. Girard (48) again took up the question in 1941 and defined the characteristics which, according to his experience with the E. V. and Tjiwidej strains had proven their value in a wide application in the vaccination of humans in Madagascar and Java, permitting one to consider a so-called "avirulent" strain as suitable for use as a living vaccine. According to Girard, a living vaccine is not truly active unless the strain from which it is derived still retains a mild virulence and a certain degree of toxicity. The demonstration of this attenuated virulence (avirulence according to Meyer) has been provided by Otten (16), Girard & Radaody-Ralarosy (49), and Walker *et al.* (50) who have observed the multiplication, more or less noticeable according to the strain, of bacteria in the animal organism during the first few days, their extremely rapid disappearance from the blood (15 min.) if the inoculation is made intravenously, and their persistence in organs such as the liver and the spleen from which they disappear after 9 to 14 days. As regards the E. V. strain (Girard and Robic), Bablet *et al.* (51) have described the intermediate reactions as demonstrated in the spleens of guinea pigs, sacrificed at various intervals after inoculation with heavy doses of this living vaccine. There are no similar phenomena when one employs virulent or avirulent bacilli which have been killed by heating to 60°. Jawetz & Meyer (52), who have studied the reactions of mice and guinea pigs to inoculation with living *P. pestis*, claim that there are only quantitative differences between the virulent and avirulent strains. The disappearance of the microorganisms from the organs after several days and the immunity which ensues leads these authors to believe that the protection conferred by a living vaccine does not depend upon the persistence of the germ, but upon impregnation of the tissues by a specific active antigen.

We do not doubt that with the insoluble fractions of Baker *et al.* the guinea pig can acquire a certain degree of immunity, but is this immunity firm and lasting? This is the real problem when one speaks of vaccinating the guinea pig against plague. Girard & Robic (53) have shown that guinea pigs inoculated with the E.V. strain were protected against very severe test doses for a year or more. It has been possible to immunize animals by a single subcutaneous inoculation against intratracheal administration of virulent bacilli (54). Twenty years after these experiments in Madagascar, Girard reproduced them at l'Institut Pasteur de Paris with analogous results. If one admits that this strain is very rich in the insoluble fraction, active for

the guinea pig, and more suitable than any other for immunizing this animal species, how can it be explained that doses 100 times higher of the same vaccine when the bacilli are dead, confer no immunity? There are two possible explanations for these experimental findings: either this antigen is so unstable that it disappears with the vitality of the strain; or it is produced in a large quantity in the organism at the time of the slight multiplication of bacilli which follows their inoculation. This second hypothesis agrees with the suggestion of Meyer (55). For us, immunization by a living avirulent vaccine, suitably chosen, at least in the special case of the guinea pig, is most closely analogous to the immunity conferred by the natural malady. In fact, in a group of guinea pigs inoculated with highly virulent cells, those animals which exhibit local and general reactions which do not result in death are subsequently strongly protected against the most severe trial infections. It is this evidence, shown many times in Madagascar, together with the absolute failure of guinea pig immunization by all types of killed vaccines; that has led us to the investigation of a living vaccine.

Finally, one remark should be made on the subject of fraction I. It is more abundant in cultures incubated at 37° than at a lower temperature. But the vaccine of the Haffkine Institute of Bombay is prepared from casein hydrolysate cultures at 28°C. according to the technique of Sokhey *et al.* (56, 57). Its protective properties are recognized for the mouse, although it is of no use with the guinea pig. We know that cultures of *P. pestis* made at room temperature are more toxic than those made at 37°, and Baker *et al.* (58) have noted the high toxicity of such cultures, extracts of which have only a low content of fraction I. Also, may one not wonder if the antigenic and toxic fraction II does not play a role in the recognized effectiveness of the new vaccine of the Bombay Institute. In truth, if the immunizing fraction is best produced at 37°, there is no reason for placing cultures at 28° for, according to Silverman *et al.* (59) and Englesberg & Levy (12a), one obtains flourishing cultures at 37° in synthetic liquid media provided the well-inoculated flasks are constantly agitated and that the pH is readjusted during growth. Similar experiments should be carried out at 28° and a comparison made of the protective power for the mouse of the two samples of antigen.

*Serological tests and anti-plague immunity.*—Man and other animals respond to the injection of *P. pestis* antigens (living vaccines, killed vaccines, or antigens from the envelope and capsule) by the production of specific antibodies. Up to what point does a measurement of these antibodies permit one to judge the degree of immunity conferred by a given vaccinal preparation? Meyer (55) has recently described the present state of this question mainly on the basis of investigations of the subject carried out by himself and his collaborators.

Sero-agglutination, complement fixation, and hemagglutination, when positive, indicate that the animal has reacted to the administration of antigen by the production of antibodies, without authorizing one to affirm that

the presence of these antibodies assures defense against experimental or natural plague infection. Nor can this be concluded from seroprotection tests in the mouse as applied by Jawetz & Meyer (60) to the study of serums from hyperimmunized animals, and by these authors (45) and also by Favarel (61) to the study of serums of subjects vaccinated against plague or of those who have recovered from plague. This test can give positive results even though the serum contains no discernible content of agglutinins and is not capable of complement fixation. Protein hemagglutination deserves particular mention because it is used in place of agglutination and fixation reactions in diagnostic practice and in research. Techniques which differ only in slight details have been described for it by Néel & Baltazard (62), Chen & Meyer (63), and Landy & Trapani (64). With fraction IA or IB as antigens which are easily fixed to tannic acid-treated erythrocytes, comparative studies of Néel *et al.* (65) and of Chen & Meyer (63) affirm the superiority of protein hemagglutination as regards both specificity and sensitivity over the polysaccharide hemagglutination of Chen (66), agglutination, conglutination, and complement fixation. The reaction is negative with anti-pseudotuberculosis sera. It would be desirable to have an antigen standardized by conclusive tests at the disposition of investigators to enable them to work under comparable conditions. But protein hemagglutination cannot be interpreted, when positive, as a certain sign of immunity. We have found it positive (Girard & Néel, unpublished) at 1-32(++) and 1-64(+) dilutions with a guinea pig serum 20 days after the injection of two doses of killed vaccine. The guinea pig succumbed a month later in a virulence test after the same interval as the non-vaccinated control; it had not benefited then from any appreciable immunity. Three guinea pigs vaccinated with E.V. living vaccine and tested three weeks later were found to be resistant. Hemagglutination titers on blood samples taken subsequently were, for one, 1-128(+) and for the other two, 1-64(+). If fraction I does not participate in the protection of the guinea pig, which is hardly doubtful according to Meyer *et al.*, it is obvious that the test in question carried out with this antigen proves by its results only that an antigen-antibody reaction is taking place, which at least for the guinea pig is not correlated with immunity. It would be interesting to test the hemagglutination of erythrocytes sensitized by the residual antigenic fraction, which is always active for the guinea pig according to Meyer, and to compare the results with those of preceding experiments. Would one perhaps find a more marked difference between animals according to whether or not they are protected after the administration of various types of vaccines?

*Mechanism of immunity against plague.*—What intrinsic factors come into play to confer immunity against plague? This problem is complex and has preoccupied investigators for a long time. Meyer (67) has clearly stated the fundamentals and proposed a solution which corresponds to the experimental findings. Whether it is a question of species such as birds, which are resistant to plague, or of those which are susceptible to natural infection and

which have become resistant after recovery or effective vaccination, phagocytosis is responsible for protection against the invasion of *P. pestis*. Anti-plague serum has no bactericidal or bacteriostatic effect *in vitro*. However, according to a study made by Hoessly in 1949 (cited by Meyer), the bacilli when subjected to the action of an antiserum undergo morphological changes which affect notably the surface, the location of the "envelope." Definite alterations can be seen with the aid of phase-contrast. These alterations in the antigen of the bacillus protective against phagocytes constitute the first stage which will permit the phagocytes to intervene effectively. *In vivo* the defense processes come into play in all animals, immune or nonimmune, as demonstrated by a series of cytograms made by Meyer (67) on serous fluid removed from the area of subcutaneous inoculation of the bacillus. However, in susceptible animals the process is only transitory and does not last more than 24 hr., after which the infection follows its habitual course; in immune ones, it is prolonged and leads to a rapid sterilization. An identical process governs the destiny in the unimmunized animal of plague bacilli which are avirulent or of attenuated virulence, the inoculation of which the animal withstands without harm. The antigenic proteins stimulate the production of antibodies in the reticulo-endothelial system, although these antibodies cannot always be revealed in the blood. They will be mobilized, in case of infection, in the inflammatory exudate and in satellite lymph nodes in the immunized man or animal.

Pokroyskaya & Kagonova (68) have contributed some very interesting data on the phagocytic power of elements of the reticulo-endothelial system and of leucocytes which are very active following inoculation of a living plague vaccine. Killed vaccines, practically ineffective for the guinea pig, do not bring about any such effects. Local vaccination, according to the principle of Besredka, definitely increases the production of clasmotocytes, normally very rare in pulmonary tissue. With repeated inhalations of living vaccine (strain E.V. of Girard and Robic and a Russian strain, A.M.P.), the number of clasmotocytes increases more than after the subcutaneous administration of the same vaccine. Meyer (67) confirmed this observation after intranasal instillation of the E.V. strain into the mouse.

In résumé, immunity to plague infection appears to be of a cellular rather than humoral nature. The specific antibodies demonstrated in the serum are in reality only witnesses of this cellular immunity, which is implemented finally by an increased phagocytic capacity of macrophages and microphages. This opinion, formulated by Meyer, will be agreed to by most authors. Furthermore, it confirms and completes the views of the early authors.

#### EPIDEMIOLOGY

The notion of a reservoir of plague infection was for a long time limited to domestic rats, but it has been progressively extended to include an ever-growing list of rodent species susceptible to natural plague or found to be

carriers of infected fleas. One counts now 186 species of these rodents, and to this must still be added 14 species of *Leporidae* as well as some carnivores, insectivores, marsupials, and primates (69). Likewise, entomologists have sought to penetrate more intimately the relationship between ecological conditions and the biology of plague-bearing fleas which constitute also, in association with rodents, disease reservoirs. The doctrine, established by the Commission of Investigation of Plague in India and which has been authoritative for so long, is challenged by serious criticisms. But let us not forget that since 1894, in all places where bubonic plague has raged or is presently raging, one has found without exception the domestic rat *Rattus rattus* or *R. norvegicus* and the plague-bearing flea most dangerous to man, *Xenopsylla cheopis*. This combination is responsible for epidemic manifestations in India, Java, Madagascar, Egypt, Senegal, Kenya, Peru, Brazil and elsewhere. On the other hand, an important disease reservoir may exist and perpetuate itself in a country without notable repercussions in man if the rat does not participate in the process. This is the case in the western part of the United States of America.

*Should the rat always be considered as a reservoir of plague infection?*—This is the question raised and discussed by Baltazard *et al.* (70, 71) from evidence gathered during a small epidemic of plague which occurred in 1947 in Iranian Kurdistan. A general investigation revealed that in those sectors of the country where one suspected the existence of a former focus of plague which had led to outbreaks in the population in 1867, an episode of pneumonic plague attracted attention in the same villages 60 years later. Rats were unknown there and one found only wild rodents, notably *Meriones*, which were shown to be infected with plague. In this species, the disease does not take the form of an epizootic; experimental infection reveals a marked degree of resistance (72). Under natural conditions, the infection assumes the aspect of a permanent enzootic, latent, or inapparent pestilence, nevertheless capable of evolving into acute mortal plague under conditions which are still obscure. As to the fleas associated with the rodents in Kurdistan, they are peculiar to the wild rodents, but can bite man; 60 per cent are of the genus *Xenopsylla*. Baltazard believes that rats which are highly sensitive to the plague are wiped out by epizootics and cannot assure that the bacillus will be perpetuated in nature; they will be, writes Baltazard, epizootic victims of the infection which they contracted by contact with sylvatic infection centers. This point of view is debatable. Very dense rat populations can be exterminated by plague as one saw in the city of Bombay, for example. But elsewhere, where the rats have at their disposition an almost unlimited living area, epizootics are the exception and definitely localized. For 30 years, murine plague has been of an enzootic character on the plateaus of Madagascar where the infection has not ceased to strike both man and rat. Contrary to the observations made at Bombay where, following the hecatombs due to the epizootics, the new generations of rats became resistant, the rats of the Tananarive district of Madagascar were just as sensitive to experimental plague in 1940 as in 1922 at the beginning of pestilential mani-



festations in that sector [Robic (73)]. Again in Madagascar, Girard (74) has noted the reawakening many years later of narrowly localized foci of murine plague, without there having been the slightest indication of a re-infection of the rat from an unfamiliar species of animal which might represent the authentic reservoir of bacilli in Madagascar. In fact, the investigations which have been carried out over a long period of time in this direction remain fruitless, and the only animals, except *R. rattus*, in which the plague has been identified in nature are guinea pigs and domestic rabbits. These rabbits have suffered epizootics in conjunction with cases of murine plague which have preceded and not followed the infection of the rabbits (75, 76, 77). One of the important results of the problem described by Baltazard would be to stimulate research along the lines he has indicated wherever the rat seemed to be the effective disease reservoir. Let us recall in this regard that at the time of the plague epidemic in Paris in 1919-1920, examination of the rats (*R. norvegicus*) revealed to Bordas *et al.* (78) a chronic form of plague, with the presence in the organs of germs of attenuated virulence which produced only subacute plague in the guinea pig. In the light of this claim, the authors entitled their article "Le rat, réservoir de virus . . .," thus emphasizing that, besides the recognized mortality inherent in epizootics, there exists another method of infection of the rat compatible with its survival which is capable of furnishing an explanation of the persistence of *P. pestis* in nature. It is not a question of underestimating the role of wild rodents as disease reservoirs in the areas of early scourges such as in Asia and Africa, but of demonstrating whether the rat constitutes an entirely independent reservoir. Bacteriology should aid this study by distinguishing the strains of *P. pestis* isolated from wild rodents and also from domestic rats in regions where these investigations are pursued. In Kenya, where the rat has long been considered the sole agent responsible for cases of plague in humans [Roberts (79, 80)], a center of wild plague was discovered in 1952 by Heisch *et al.* (81). In the opinion of Girard (82) this source, linked to those of Central Africa, is distinct from those which the rat itself has created after the importation of the oceanic variety to the African coast since 1894. This has in fact confirmed the stability of the essential biochemical characteristic (glycerol+) of strains of wild plague organisms in Kenya after 50 passages through the rat. But all the strains of *P. pestis* isolated until that time from rat or man in endemic urban areas where the principal epidemics in Kenya have occurred for the last 50 years are of the oceanic type (glycerol 0). Sources of wild and of murine plague are then absolutely independent of each other. On the contrary, plague affecting *Citellus* in California appears to be secondary to murine plague imported at the end of the last century because the strains are of the oceanic type. It constitutes a disease reservoir independent of the rat, which behaves like that of the merions in Iran for, according to Meyer & Eddie (83), one found in 1936 squirrels with infected fleas in burrows in two areas distinctly located where *Citellus* was known to have had plague 20 years before.

*The fleas of rodents, reservoirs of plague infection.*—This notion has been



confirmed with the discovery of infected fleas living independently of their hosts. The longevity of these "free" fleas is strictly dependent upon climatic conditions, upon the existence of subclimates in which the principal factors are temperature and humidity. This is a very complex problem in which the data vary with each species of plague-bearing flea. Some significant studies of this subject are those of Burroughs (84), Eskey & Haas (85), and of Wheeler & Douglas (86). In the plateau region of Madagascar, the climatic conditions are favorable to the survival of *X. cheopis* in the free and infected state as reported by Girard & Estrade (87, 88). They think that the continuance of enzootic and endemic pestilence is linked to the particular conditions found in this high region. Outbreaks of human and murine plague in the hot coastal regions, on the other hand, are only transitory since the climate there is not favorable to the viability of this species, which moreover is rare and dies rapidly when liberated by the death of its normal host. George & Webster (89) in the Cumbum Valley of South India and Macchiavello (90) in Brazil have made observations which corroborate those made in Madagascar on the role of *X. cheopis* in the persistence of the plague. Finally, the notion of efficiency of the plague-bearing fleas as vectors has resulted in the discovery of considerable differences between species. This idea, established by Wheeler & Douglas (91) has been applied by Devignat (92) in the Belgian Congo, and then by Holdenried (93) in the United States in his study of *Diamanus montanus* and *Polygenis gwyni* compared to *X. cheopis* which is considered by that author as the most powerful vector. Furthermore, these results compared with those of Wheeler & Douglas (86) led Holdenried to conclude that variable and unknown factors intervene in the quality of plague-producing potential of a particular pulicose species. There is still considerable obscurity in this field.

*Man and human ectoparasites in the epidemiological complex.*—Blanc & Baltazard (94, 95) have evaluated the role of human ectoparasites, particularly of *Pulex irritans*, in relation to the present state of knowledge of plague epidemiology. According to these authors, there can be no epidemic without inter-human transmission in which these ectoparasites are of foremost importance. Girard (96) has discussed this point of view at length. In Morocco, special circumstances helped support the thesis of Blanc & Baltazard, but it has not been accepted as being generally applicable. However, in emphasizing the possibility of fleas contracting the infection from man in the septicemic stage of the disease, these authors have in this way implicated the plague-bearing species capable of biting man as a causative agent. From what we said above about the "free" murine fleas, in particular *X. cheopis* which sometimes grows prolifically in the dust of native huts (87), man is then capable of inclusion in the epidemiological process. For the classic symbol "Rat-Man," representing the transmission of the plague to man only by the fleas of rats, is substituted a four link chain, "Rat-Man-Man-Rat," in which man infected first by a rat's fleas can in turn infect his habitual ectoparasites (fleas and lice) or occasionally *X. cheopis*, the latter

being capable of reinfecting the rat. This formula takes account, in an acceptable system, of all the facts observed in endemic territories where climatic factors and other local conditions, especially those connected with the manner of habitation and the degree of evolution of the population, are responsible for such a diversity of aspects.

#### PHOPHYLAXIS

*Etiological prophylaxis.*—The battle against the rat has long been the dominant preoccupation. It retains its importance, but logically gives way, in case of an emergency arising from an epidemic or the menace of an epidemic, to the battle against the vector agent. It has been proven that the systematic killing of insects by modern insecticides brings about a spectacular diminution in the pulicose index, and, by consequence, a rapid cessation of cases of human and murine plague. A rigorous technique should prevail in these operations if one wishes to obtain the maximum efficiency, as has been reported by Macchiavello (97) at the time of the epidemic in Tumbes (Peru) in 1945 which was allayed in two weeks. In India [Wagle & Seal (98)], in Madagascar [Mercier (99)], and wherever it has been possible to control insects with the desired means, *X. cheopis* has been almost completely exterminated with the subsequent disappearance of human and murine infection. Control of rats remains a measure of secondary urgency in time of epidemics and is a perennial concern in the latent periods. This project meets with serious difficulties, especially in rural sectors of relatively unevolved countries, and it is precisely there that plague is firmly rooted and undergoes periodic revivals. Wherever the offensive attack against the rat is possible, the use of anticoagulants is recommended (100). As to control of wild rodents, one should not be lulled by illusions; it will produce only aleatory results. The method to be followed has been discerningly decreed by Davis (101): locate and keep under close surveillance the centers of sylvatic plague in order to prevent transmission of the infection to commensal rodents and to human beings living in the area.

*Personal prophylaxis. Vaccination.*—The last 20 years have been distinguished by the use of living anti-plague vaccines on a previously unknown scale; in fact, millions of inoculations have been performed without the slightest mishap with two strains of *P. pestis* of attenuated virulence: the E.V. strain (Girard & Robic) in Madagascar, and the Tjiwidaej strain (Otten) in Java. Because of the small measure of success realized with killed vaccines, these authors were led to recommend this new method of immunization after an experiment which proved that the guinea pig could not be given lasting protection except by living bacilli. This question was discussed earlier under immunology. The efficacy of these vaccines has been demonstrated by their use under circumstances having the value of a veritable human experiment in Java (16, 102) and in Madagascar (103, 104, 105). Without any measure other than vaccination, the number of cases of plague in the epidemic period was reduced by more than 80 per cent. Grasset (106)

in the Union of South Africa and Devignat (107) in the Belgian Congo have also used living vaccines successfully. These require but a single inoculation, whereas the killed vaccines, whether one employs the whole organism or antigenic fractions, require at least two injections in order to confer an appreciable immunity. The living vaccines then have the advantage for mass vaccinations that a widely-dispersed population need be assembled but once during a campaign. But whatever vaccine is employed, immunity is lessened after several months and it is recommended that the inoculation be repeated annually for several years in certain hyperendemic sectors such as exist in Madagascar. Nevertheless, the living vaccines are inconvenient since they cannot be stored; they must be prepared only as needed as their effectiveness does not exceed three weeks, even when kept at about  $+5^{\circ}\text{C}$ . Control of the properties of the vaccinal strains should be constant, not so much for fear of a recurrence of virulence as to insure against the loss of antigenic power as has happened in many laboratories with the E.V. strain when kept under unfavorable conditions. Conservation and distribution of comparable strains, properly controlled, should devolve upon official laboratories competent in this procedure. The chemical vaccines (antigens of Baker *et al.*, of Amies, and of Seal) have not advanced beyond the experimental stage.

Should one consider vaccination as an outmoded measure which will disappear with the successful progress of causal prophylaxis? Such is not our opinion. Insect control by contact insecticides with lasting action has, as we have seen, rightly taken the preeminent place in the battle against plague. But already certain limitations have been noted as to the permanence of anticipated results. Säenz Vera (108) reported that in 1952 in rural sectors of the state of Pernambuco, Brazil, after several years use of DDT, *X. cheopis* showed a certain degree of resistance to this insecticide. This finding has seriously concerned the sanitary authorities. Also, it would not be prudent to neglect any aspect of prophylaxis. Just as insect control benefits from investigations which exploit the immense resources of synthetic chemistry and led to the preparation of new and more active compounds—in this regard a recent report by Ryckman (109) is very suggestive—so research directed toward the perfection of antiplague vaccination should not be interrupted.

#### THERAPEUTICS

Until 1938, specific serum therapy was the only treatment for plague. Its effectiveness, limited to the bubonic form, was constantly debated; it could not be otherwise, for the seriousness of the malady varies considerably from one center to another. It is difficult to compare the efficacy of an identical treatment applied under noncomparable conditions: in one country the over-all mortality for the untreated cases figures between 20 and 50 per cent and for another it is in the neighborhood of 100 per cent.

First, the sulfonamides and subsequently the fungus antibiotics have

everywhere completely transformed the prognosis for plague in its various clinical manifestations. One is justified at present in stating that any case of plague should be rapidly cured without complications if it is treated in due time. In practice, the sulfonamides such as sulfathiazol, sulfadiazine, and sulfamerazine, are alone sufficient to cure light or moderately severe cases of bubonic plague. In septicemic cases and for pneumonic plague, streptomycin is incontestably the preferred medicine which we have qualified as "heroique." But chloramphenicol (chloromycetin) and oxytetracycline (terramycin) also have much success to their credit. There is copious documentation on the experimental work accomplished in this field in the last 16 years, as well as of results obtained from applications of these agents to the treatment of human plague, in India notably for bubonic plague, and in Madagascar for pneumonic plague. The reader will find in several publications, which are rich in bibliographic references [Meyer *et al.* (110), Smadel *et al.* (111), Pollitzer (112), and Girard (113)], the list of these works. Among the authors who have made notable contributions to this work in the laboratory as well as in the field let us mention Sokhey, Wagle and Habbu in India, Estrade, Mercier, Robic, McCrumb, and Favarel in Madagascar, Devignat in the Belgian Congo, Roux and Mercier in Algeria, Meyer in San Francisco, Girard and Néel in Paris, Schütze in London, and Durand and Magrou in Tunisia. The most spectacular effect of anti-plague chemotherapy is that observed with pneumonic plague which was irremediably fatal in Madagascar, as it was also at the time of the great Manchurian epidemic in 1911, before the advent of streptomycin. The observations compiled by Robic (114), together with the results of bacteriological examinations which accompany them, are most instructive. About 10 hr. after the beginning of treatment virulent forms in the sputum disappear, as a consequence of which the risk of contamination of the attendant staff and of those associating with the sick person is rapidly reduced. A total of 15 to 18 gm. of antibiotic administered in 6 days constitutes the usual curative dose. One associates the sulfonamides with an extended range of action; sulfadiazine for example is administered in a dose of 2 gm. per day for 10 to 15 days to prevent the development of "substitution" microorganisms (Brisou) capable of provoking complications, as was the case in Madagascar where a victim of pulmonary plague succumbed to a streptococcal lung abscess even though the plague bacillus had long been destroyed (113). Finally, the administration of sulfamides (3 gm. per day for a week) appears to reduce the number of cases of pneumonic plague among people who are in contact with sufferers from plague capable of spreading the disease by expectoration. Even though there were some failures in Madagascar (115), the passage of time and the development of this prophylactic method now permit one to affirm that it is efficacious on condition that it is instituted without delay. Finally, let us emphasize that penicillin is without effect on *P. pestis in vitro* as well as *in vivo*.

Should specific serum therapy be deliberately abandoned in view of the

success of chemotherapy? Girard (116) does not think so. Anti-plague serum differs in mode of action from antibiotics: the latter are antibacterial; the serum is antitoxic. Clinical findings and experimentation justify this interpretation. While antibiotics are capable of curing the majority of cases of plague, the sulfonamide-serum association has given superior results in Madagascar and in the Belgian Congo [Devignat (117)]. However, the authors are not of unanimous opinion on this point. For example, Sokhey & Wagle (118), although recognizing that the sulfathiazole-serum association is better than sulfathiazole alone, report that sulfadiazine without serum has given a higher measure of success. Girard agrees with Meyer that it is in the hypertoxic forms of plague infection that there is an advantage in combining the two medications. Some recent observations of Devignat [not published, cited by Girard (116)] are significant as regards this subject. Serum therapy as adjuvant medication would also be indicated when treatment is late in being instituted and there is fear of a massive liberation of toxin under the influence of intense microbial lysis provoked by streptomycin, which is not only bacteriostatic but also bacteriolytic. Also, the recent studies of Englesberg & Levy (119) on the production of a particularly active toxin when *P. pestis* was cultivated in a liquid medium—casein hydrolysate with the addition of glucose and inorganic compounds—should be taken into consideration. With this purified concentrated toxin, one can envisage the preparation of a serum of high antitoxic power, of the type of gamma globulin, already obtained from the rabbit by Meyer *et al.* (110), but which circumstances have not yet permitted to be used in treatment of human plague.

#### PRESENT STATUS OF PLAGUE THROUGHOUT THE WORLD

*Prospects for the future.*—The modern pandemic is declining. The contrast is striking when one compares the statistics for plague mortality in India in the last two decades with those of the period from 1900 to 1930, to consider only the country which has paid the highest toll to this scourge. This situation is legitimately attributed to the success of a prophylactic method, the result of fruitful research which has followed the fundamental discoveries of Yersin and Simond; but one must also include the contribution of natural circumstances in order to explain this regression in countries such as Uganda where the control has been too limited to be effective, as emphasized by Davis (120). The pestilence curve, the general outline of which indicates a descent, shows some peaks which remind us that in many parts of the world aggressive disease sources still persist. Not the least important achievement of recent work has been to expose the importance of the reservoir represented by wild rodents which, according to Pollitzer, may not have ceased to expand into new areas. But the facts give evidence that this "wild" plague does not directly menace man as long as commensal rodents are not affected. If the rat does not constitute an independent reservoir of plague infection, the major preoccupation in territories affected with this

"wild" plague should be to eliminate the passage of the bacillus to the rat. This, for example, is the present situation in the western part of the United States of America.

Considered from the angle of the perpetuation of plague bacilli in nature, and without considering the part contributed by each animal species, climatic factors play the most prominent role in this regard by way of their influence on the behavior of plague-bearing fleas as demonstrated in the work of Davis (120, 121) on the plague in African territories. As to the problem of human plague, it amounts essentially to a question of habitation. It is practically solved in countries of advanced civilization henceforth secure from the great historical epidemics. The 20th century pandemia has nevertheless branched out to new endemic foci of which certain ones in America, Africa, and Madagascar appear to be rather permanent. With the development of means of rapid transportation, should the slightest negligence intervene in the execution of international sanitary police measures, plague could be introduced into all parts of the world; but it would quickly be conquered by the prophylactic and therapeutic measures at our disposal. Such was the case in Paris in 1919, and in Corsica, Naples, Malta, Palestine, and elsewhere in the last decade. The situation is different in rural sectors of under-developed countries which, in the matter of habitation, are still in the era of medieval cottages. In these places, one needs constant vigilance and a system of control which cannot easily be standardized because of many local considerations among which psychological factors are not negligible. Success will be obtained only with the cooperation of the people and not by force. Tracking down cases of plague should be one of the first considerations, and it is the most difficult task to accomplish. Actually, one only learns of the incidence of human plague in a particular section by resorting to a systematic search for dead bodies, as Girard (122) proved in Madagascar. But the nearly certain assurance of a cure with existing medication and the value of effective vaccination recognized by the population will little by little triumph over their resistance to medico-prophylactic measures.

What are the future prospects of this scourge whose name always inspires terror? Fabian Hirst (123) has just written a splendid book under the title *Conquest of Plague*, equally rich in science and instruction and which, by its sub-title, "A study of the evolution of epidemiology," extensively covers the subject. We have conquered plague as an epidemic scourge, but the source of harm remains because it is external to man and its total eradication would be utopian. One asks also why plague has always assumed a short- or long-term periodicity in its local or global manifestations. In spite of the progress of our knowledge we are reduced to invoking the "Génie Epidémique" of the ancients, a picturesque expression indeed, which in fact hides our ignorance. Modern authors tend to see fluctuations of rodent populations as the reason for this cyclic behavior of plague; they obey a periodic law which, according to Elton (124) is controlled by the cycle of



sun spots. The immunity acquired by animals which survive epizootics and which is temporarily transmitted to their descendants also enters into the balance. Fabian Hirst (125), who has devoted a long chapter in his work to this stimulating problem, recalls that in 1881 Pasteur *et al.* (126) suggested that the causative agent, which was not discovered and identified until 13 years later by Yersin, might persist in nature in an attenuated form with a sudden return of virulence controlled by climatic or economic factors. It was in regard to the plague epidemic which struck Bengâsi (Lybia) in 1851 that this totally intuitive hypothesis was pronounced by Pasteur. With the passage of time, it appears justifiable if one takes into consideration the existence of strains of *P. pestis* of attenuated virulence, in rat tissues (78) notably, and whose destiny is not known to us. But we now know to what extent bacteriophages can create multiple variants of *P. pestis* by reason of the high sensitivity of this microorganism to their action and of the late appearance of secondary cultures. Is it not possible that these variants obtained *in vitro*, whose virulence is always weak or negligible (29), can be produced naturally during an epizootic which liberates in the soil and water, masses of microorganisms? These germs will be contaminated with phages existing in soil and water, for the specificity of the phages is not indispensable because numerous enterobacterial phages have a lytic power affecting the plague bacillus. Cannot a mutation process also cause a recurrence of virulence in certain attenuated organisms, analogous to that which was twice observed by Girard (30) with mutants of the E.V. vaccinal strain which had lost the characteristics of the mother strain from which they were derived? In the perpetuity of the "complexe pesteux" [Sorre (127)], one is above all concerned with the behavior of disease reservoirs and only slightly with the etiological agent, as if the latter should immutably retain the properties classically associated with it. In considering the intervention of bacteriophages, we follow the path indicated by d'Hérelle (128), who emphasized the importance of mutants in cholera epidemiology and formulated the hypothesis of a rupture of the "vibrio-bacteriophage" symbiosis, with return to the typical vibrio form pathogenic to some host peculiar to the endemic zone (Bengal) where the epidemics always start. Gaiski [cited by Pollitzer (129)] assigns to bacteriophage a role in the evolution of plague infection with relation to tarabagans, but Pollitzer has not been able to confirm the presence of lytic agents associated with these animals. Be that as it may, this working hypothesis should provide an incentive for research which up to the present time has only been outlined, and which in our opinion is full of promise.

We cannot conclude this exposition without calling the loimologist's attention to Pollitzer's substantial work, *Plague*, to which we have so many times directed the reader in our bibliography. This exhaustive work on plague in all its aspects shows the path travelled since 1936 when appeared, under the same title the "Manual" edited by Wu Lien-Teh, Chun, Pollitzer and Wu.



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## THE COXSACKIE VIRUSES<sup>1</sup>

BY GILBERT DALLDORF

*Division of Laboratories and Research, New York Department of  
Health, Albany, New York*

The proposition that the Coxsackie viruses (1) can be recognized by their unusual pathogenicity for and lesions in suckling mice (2, 3) has survived with remarkably little qualification. The importance of separating these agents into two groups, A and B, (4) has been apparent in numerous studies and it is now generally accepted that the distinction is valuable both to the clinician and the microbiologist. The classification proposed in 1949 (5, 6), which is based on groups designated by capital letters and types identified by Arabic figures, is widely used and was endorsed by an Ad Hoc panel under the chairman of the Virus Subcommittee of the International Committee on Bacteriological Nomenclature, meeting during the sessions of the International Congress for Microbiology, Rome, 1953.<sup>2</sup>

Several questions have been raised and answered regarding the validity of the proposed grouping and the significance of the lesions and the age effect. "Strains" that have induced anatomical changes resembling those of both groups have in our experience invariably proved to be mixtures of viruses belonging to both groups. No strains received from other laboratories or isolated by us have thus far been ambiguous regarding grouping. The diagnostic significance of the lesions of the fatpads has, however, been modified by the observation of Schwartzman that poliomyelitis virus causes similar effects in animals that have been dosed with cortisone (8).

The reported discrepancies respecting pancreatic lesions were resolved with the demonstration that tropism for the pancreas is indeed a common property of freshly isolated Group B strains but one that may be irrevocably lost if the virus is repeatedly passaged as infected brain rather than carcass suspensions. Pancreatic tropism is intensified if infective pancreatic tissue is the source of the inocula. The pancreatic lesion appears to be specific at present but may be lacking in the case of some strains and after repeated transfers of brain suspensions (9).

The susceptibility of only the very young to infection with Coxsackie viruses may now be more precisely defined. In the isolation of these viruses very young mice alone will serve. Indeed for the isolation of the Group B strains, it is best to use animals younger than two days. However, many other viruses are much more infectious and pathogenic for infant than adult mice and it may well be that some can at times be isolated only by taking

<sup>1</sup> The survey of literature pertaining to this review was completed in December, 1954.

<sup>2</sup> de Góes and Travassos (7) propose that all Coxsackie virus infections be spoken of as "coxsackioses."

advantage of the greater susceptibility of infant animals. Furthermore, other viruses may yet be found that equal the Cocksackie family in the degree to which they are dependent on immature mice. The surveys underway in Egypt and India (10, 11), in which newborn and mature mice are used as test animals, promise to add further information on this point.

Moreover, it is true, and has been known for some time, that a degree of susceptibility persists in older animals. Pancreatotropic strains may cause lesions in mature mice (12, 13) and certain Group A strains, foci of muscle degeneration (14). Severe illness and death are infrequent in both cases. Such infections can be intensified by administering cortisone (15, 16). Recent work in our laboratories suggests that adrenocortical activity may somehow be related to the age susceptibility of mice to Cocksackie virus infection. The evidence consists of the demonstration that cortisone does not increase the severity of disease or the titer of virus in the tissues of very young animals. The effect of cortisone becomes apparent as natural susceptibility wanes and is most marked in adults. If the observations are correlated with what is known of the size and activity of the adrenal cortex at these ages, one is led to suspect that newborn mice are spontaneously subject to intense adrenocortical hormone effect and that, as the cortex involutes with age, susceptibility may be restored by extrinsic hormone. The clues we have point to maternal hormone effect immediately after birth, followed by a high level of cortical activity in the young and waning activity thereafter.

Cheever (17) determined the effect of whole body roentgen radiation on the susceptibility of adult mice to the Powers strain (Group B, Type 4) of virus. Virus could be demonstrated over a longer period of time in the organs of the irradiated mice and, with rare exceptions, at a higher titer. There was, however, an irregular and only moderate increase in mortality among the animals that had been irradiated.

Wallis & Sulkin (18) report that 15-gram mice were fatally infected with two strains of Cocksackie virus when urethane was added to their drinking water. They tested eight strains. The two that proved to be active, Conn. 5 and Nancy, are members of Group B. Conn. 5 is a Type 1 and Nancy, a Type 3. The six that failed to infect were not identified. The B3 strain was passaged serially.

Detailed and comprehensive studies of the age susceptibility of mice to Cocksackie virus infections have been reported by Howes (19, 20). All but one of the six Group A strains examined were qualitatively similar. The exception, Type 1, is well known to differ in titer (lower) and incubation period (longer) and possibly in its physical properties (21). It has not been associated with herpangina and neutralizing antibodies are not as frequent as is true of many Group A strains (22). Howes noted that paralysis was more spastic and appeared later in mice infected with Type 1 than with the other types. He found the initial phase of susceptibility to Group A infections persisted to the tenth day, after which it declined, first slowly and then precipitately, to be lost during the fourth week. The circumstances regarding

the Group B strains were more complex. The initial susceptibility fell rapidly but rose to a second peak during the third week. This fits the earlier observation that the susceptibility of the mouse pancreas disappears by the fourth day of life but returns after several weeks (23). The various Group B strains studied differed considerably among themselves, but it was not clear whether this was due to inherent or acquired differences. Howes verified the early and higher titer of virus in the pancreas following Group B infections. His study has the additional significance of providing a further striking distinction between Coxsackie viruses of the two groups.

Currently more attention is being paid to the Group B than the Group A Coxsackie viruses. This may be attributable to a growing realization that the Group B strains are more important as agents of disease in man and to the fact that they are as a group more readily isolated by tissue culture technics. Group B strains of all the known types are cytopathogenic on cultures of the trypsinized monkey kidney cell (24), which are now so widely used in the isolation of enteric viruses. The preliminary incubation period is in general longer than with the poliomyelitis viruses and the end-point titer is somewhat lower but they nevertheless commonly intrude in the search for poliomyelitis viruses. Only five of the 19 Group A types are cytopathogenic (25). Of the more commonly encountered types of Group A, Type 9 has been isolated in tissue cultures on several occasions (26, 27, 28). Thus, on the basis of what is so far known, there may be a considerable difference between the groups in their behavior in tissue culture and, while qualifications may come with time and variety of experimentation, it seems likely that the Group B strains will intrude more than the Group A ones in the study of poliomyelitis.

#### CLINICAL MANIFESTATIONS OF GROUP B INFECTIONS

The importance of the Group B strains as agents of disease has become increasingly clear. Fruitful observations have come from studies of aseptic meningitis. It will be remembered that Gsell (29) first noted an association of aseptic meningitis and epidemic pleurodynia. His observations were made before laboratory confirmation was possible and did not exclude the possibility that the meningitic signs represented a second infection. This has been resolved by the work of Johnsson (30) and Gard (31) who studied outbreaks of aseptic meningitis and Bornholm disease in Sweden. Their observations leave little doubt that the cases of aseptic meningitis they examined were due to a Group B (Type 3) virus. It was recovered from 45 per cent of the patients, neutralizing antibodies were regularly present during convalescence, and a rising titer of antibodies was found in all instances in which the first specimen had been collected within four days following onset of symptoms. As Gard has said, the chain of facts "seems to provide evidence as conclusive as has been produced for any other virus infection."

Johnsson noted that pleurodynia was the common symptom of adult infections, and aseptic meningitis, of infection in children. Gard postulated



that the introduction of a Group B virus into a population with previous experience might result in an epidemic of aseptic meningitis among children while in a nonimmune population adults would also be susceptible and the epidemic would resemble pleurodynia. It should be noted, however, that in the one large outbreak of Coxsackie virus infection in a nonimmune population (nonimmune on the basis of humoral antibody level) of which we have record, the illnesses were characterized clinically as encephalitis although more than half of the patients were adults. This epidemic occurred in Sydney, Australia, in 1951 (32). Headache, fever, and stiff neck were present in almost all of the hospitalized patients, muscle pain and sore throat in half. Virus was recovered from half of those examined. The epidemic was notable also for the frequency of sequelae (63 per cent) and the prolonged period of hospitalization. The responsible agent is neutralized by serum of Group B, Type 3. Thus, the Australian epidemic resembled aseptic meningitis rather than pleurodynia; the signs of encephalitis do not appear to have been prominent.

Gard also made the interesting suggestion that the syndrome described by Wallgren may be a disease, a nosologic entity, in Sweden. It is well-known that in other places and times aseptic meningitis has been caused by various viruses. Recent studies in Minnesota (33) are a good example, as are the analyses made by Adair, Gauld & Smadel (34). Gard suggests that the circumstances in Sweden are somewhat different and notes that Wallgren himself considered aseptic meningitis to be a new disease and that its growing importance and frequency parallel the evolution of epidemic poliomyelitis which also became manifest for the first time in the Scandinavian countries.

The etiologic role of Group B viruses in aseptic meningitis has been further established by the growing number of isolations from cerebrospinal fluid. The first of these was made in Gard's laboratory (35). Johnsson's study provided another. Gear (36) and Goldblum (37) report several from South Africa and from Israel. Isolations have also been reported by Rhodes (38). Gear has undertaken laboratory tests on all cases of aseptic meningitis admitted during a three-year period to the Johannesburg Fever Hospital. The cases were studied in association with Dr. Philip Bayer and his staff. Eight proved to be cases of mumps, two of herpes, and thirteen of Coxsackie virus infection. Of the latter, seven were due to Group B strains and in two of the seven the isolation was from the cerebrospinal fluid. The etiologic agent was not established in the majority of the cases (39). The importance of isolations from the cerebrospinal fluid lies in the proof of infection of the central nervous system rather than mere carriage of virus. As mentioned subsequently, Gear has also isolated Group B viruses from the nervous tissue of fatal cases.

The most recent investigation of cases of aseptic meningitis or meningo-encephalitis has been reported by South African physicians (40) who encountered five patients admitted during an outbreak of Bornholm disease with headache, muscle spasm, stiff neck, and in four of the five increased cere-

brospinal fluid cells. Group B viruses were isolated from the feces of all five. The South African workers also commented on the ill-defined nature of pleurodynia in the very young and stated that Group B Cocksackie viruses are "one of the commonest causes of this condition [meningo-encephalitis] in this part of the world."

Interestingly, Group B strains have also been implicated in cases of severe and at times fatal disease in infants, thus paralleling the events in mice. The original observations were made by Gear who investigated several small outbreaks of myocarditis in infants (41). The first occurred in Johannesburg in late 1952. A number of babies became ill while in or soon after discharge from the maternity home in which they were born. Six of the ten affected infants died after an acute, fulminating illness that ended in circulatory collapse. Tachycardia, enlargement of the liver, and slight edema of the feet were observed. The three fatal cases examined post-mortem had severe, acute myocarditis. The brain tissue of two of these yielded a Group B Cocksackie virus as did the feces. The strain proved to be Type 3. Gear subsequently studied two other small outbreaks of similar nature and from one recovered another Type 3 strain. The third yielded a Group B, Type 4 virus. Dr. Sidney Kibrick has recently isolated a virus from the tissues of an infant who died on the fifth day of life (42). Myocarditis was demonstrated in the infant's heart as well as the hearts of suckling mice inoculated with the virus. This strain was also Type 3. The association of the same type with myocarditis in infant mice had previously been observed by Kilbourne (43). Myocardial lesions were found by Dalldorf & Gifford (44) in three of fifty-one Group B mice in which the hearts had been adequately examined, and have also been described by Godman, Bunting & Melnick (23). A few attempts we have made to induce myocarditis in very young monkeys with this virus have thus far failed.

Orchitis seems to be a relatively frequent complication of Bornholm disease. Although Sylvest (45) did not personally encounter such cases, he referred to others who had, including Petrén who saw 25 patients with orchitis. Of 30 males examined by Jamieson & Prinsley (46), 12 had orchitis. It was the commonest complication they encountered. The importance of orchitis was also emphasized by Morrison & Baird (47) and by Warin and his associates (48). In the Oxford epidemic, three of 30 adult males developed orchitis on the fourteenth or fifteenth day of illness. Orchitis has usually occurred at about this time and generally has been associated with clinical relapse.

The epidemic which swept over much of Europe in 1951 was the first extensive one to be widely studied by laboratory means and a good deal was learned of unusual forms of the disease which had become recognizable for what they were by isolation of the virus or humoral studies. Thus, Disney *et al.* (49) described four kinds of atypical cases, those with symptoms of encephalitis or meningitis of which they saw seven, and those with pyrexia of unknown origin (PUO) of which they had ten. In the second group ab-

dominal pain, vomiting, or headache with sore throat and lymphadenopathy were common associated symptoms. The third group consisted of four patients with evidence of pneumonia as well as Bornholm disease, and their fourth group consisted of infants in whom screaming attacks, fever, and malaise were the usual manifestations.

It is interesting also that physicians have recently been led to characterize some cases much as Daae did in 1872 (50). Thus, McConaghey (51) suggested that an epidemic of fibrositis may have been part of the 1951 British epidemic of Bornholm disease. Many of his patients complained of vertigo with headache of sudden onset usually coming on after waking in the morning. The two most constant signs were headache and tender points in the occipital region. This simply emphasizes what many others have noted. Kilbourne's patients, resident physicians, had muscle tenderness, pains of an aching nature usually described as muscular, involving the eyes, neck, back, head, legs, or shoulders. Four had soreness of their throats. These illnesses simulated infectious mononucleosis, acute appendicitis, sinusitis, pleurodynia, or influenza. They were proven to be infected with Group B Coxsackie virus (52). McConaghey's patients were not investigated by laboratory means.

Cases of myalgia persisted in the Bretagne area of France following the 1951 epidemic and two patients were intensively studied by Lépine, Desse & Sautter (53). The first was a 61-year-old female with sudden onset of violent pain of the right knee and muscles of the right leg. The knee reflex was absent. A bit of muscle from the point of greatest tenderness showed severe degeneration of the fibers. From it and the patient's stool a Coxsackie virus was readily isolated. The second patient was 25 years of age. Having completely recovered from an attack of Bornholm disease during September, the patient relapsed later in the year with similar muscle pains. Inflammation but not degeneration was observed in the biopsy which again proved infectious for mice.

Ritchken & Gelfand related an outbreak of an acute febrile illness characterized by fever of from five days to four weeks' duration, splenomegaly, and slight lymphadenopathy to a Group B Coxsackie virus infection. The virus was recovered from five of the six cases investigated. Heterophile antibodies were not present. Atypical monocytes were seen in some blood films (54).

Mitchell & Dempster (55) described the illness of a 7-year-old girl with herpangina in whom shallow ulcers were found dorsal to the labia. They disappeared abruptly as she recovered from the herpangina. Tests for herpes virus were unfruitful. An A 10 Coxsackie virus was isolated from the throat, the feces, and the genital lesion and a rise in antibodies was demonstrated.

As laboratory aids in diagnosis become more generally available, it is likely that the protean manifestations of Coxsackie virus infections can be further analyzed and clinical criteria sharpened. Meanwhile we may summarize at this time by saying that the causal relationship between the Coxsackie viruses and herpangina, Bornholm disease, and aseptic meningitis

may now be accepted as established and the relationship of the Group B viruses to severe disease in infants is indicated. In the case of the first two diseases, the evidence suggests that Coxsackie viruses are the sole cause and that the clinical manifestations are specific and diagnostic of Group A and Group B infections respectively. Aseptic meningitis, on the other hand, is an expression of a great many infections and there are thus far no clues to a form characteristic of Coxsackie virus infection. The nature of the myocarditis of infants requires additional observations to determine whether that disease has pathognomonic features and constitutes a nosologic entity.

Prior to the identification of myocarditis neonatorum Coxsackie virus infection had never been proven to be an immediate cause of death. During the large epidemics in Denmark in the early 1930's a number of fatalities were reported but the individuals were of advanced age or had unrelated complications such as pneumonia or angina. Quinn described a fatal case in 1951 (56). The patient, a 55-year-old male, suddenly developed pleurodynia and headache. The symptoms other than headache slowly improved for several days when they were exacerbated, and vomiting, photophobia, and stiff neck were noted. The patient died four days later. Bornholm disease was epidemic in the area at the time but laboratory tests were not undertaken nor was the man examined post-mortem.

During the 1951 epidemic in Europe, two females in the fifth month of pregnancy aborted during or shortly after an attack of pleurodynia (57). Mice become progressively more susceptible to Group B Coxsackie virus infection as gestation advances and many of the litters are lost (58). The heightened susceptibility of gravid females to poliomyelitis is well-known.

#### FAMILY INFECTIONS

The frequency of multiple infections within families or small groups has sometimes been striking. We examined fecal specimens sent us by a physician who sickened while traveling by car with his family. A Group A, Type 2 virus was isolated from the father and all five children. Only the mother was uninfected. Johnsson investigated eight families in each of which he had identified an infected individual and found multiple infections in each case (59). In three of the families Group A strains were responsible; in the other five, Group B strains. Illnesses occurred in 80 per cent of the family contacts. All but one of the dozen infected with Group A strains experienced minor symptoms of one kind or another. Of those infected with the Group B strains, 16 of 23 developed aseptic meningitis, pleurodynia, or myalgia. Johnsson's experience is important in showing that, under the conditions existing at the time of his study, Coxsackie virus infection was clearly not as inconsequential and commonplace as others have at times suggested.

Sylvester (60) reported an explosive outbreak in two nearby households under common ownership. Two illnesses were described in detail. Interestingly, one was a cook and the second a food handler. Kenyon, Macrae, Dodds & Galpine described an outbreak in a children's home (61). The popu-

lation included 60 young children and a staff of 23. Of 73 residents 52 were affected as well as a number of the non-resident staff. The epidemic lasted for three to four weeks and the etiologic agent was firmly established. Smith witnessed an extensive, almost explosive epidemic of Bornholm disease in Singapore (62). More than 70 cases occurred within 24 hours among European naval personnel who, although they lived in scattered quarters, had a common mess. Smith's observation is similar to what has happened in the tropics when North American and European adults experienced poliomyelitis virus infections and it may be that the level of Coxsackie as well as poliomyelitis virus infection in the tropics is relatively high. The studies in Egypt, India, and South Africa support that view (63, 64, 65). Indeed we have the impression that infection is more common and the level of infectivity higher in the southern than the northern United States.

#### MORPHOLOGIC STUDIES

The morphologic phenomena associated with Coxsackie virus infection, first described in histologic terms, have since been defined by other technics. The muscle lesions following infection with Group A strains are most suitable for such analyses since the changes are uniform in distribution and rate of evolution. Aumonier (66) undertook to characterize the earliest changes and found that the sequence of events in striated muscles was loss of "H" and "Z" lines, loss of "A" and "I" discs, swelling of the myofibrils, and, finally, complete loss of internal structure. He postulated that Group A virus may have a specific affinity for one or more of the constituents of the myofibrils. Kausche & Hoffman-Berling (67) applied histochemical reactions and the Caspersson technic and found characteristic effects of infection within 24 hours. The changes consisted of dense perinuclear zones that spread outward and sometimes formed confluent masses. The spectroscopic absorption curves indicated that these areas consisted largely of nucleoproteins. The nuclei themselves contained increased amounts of RNA that appeared to arise from spherical nucleic-acid-containing bodies adjoining the nuclei. The signs of increased protein synthesis disappeared as the stages of degeneration visible with the light microscope became apparent.

Gädeke & Waltenberger have provided additional evidence of changes that antedate those visible microscopically. They demonstrated a reversal in the Na-K ratio in infected muscle within 30 hours following infection (68). Preliminary studies by means of the electron microscope have also disclosed earlier lesions (69) than those visible in the light microscope and promise interesting new information regarding the site and mode of action of the Group A viruses. The presence of large amounts of alkaline phosphatase in the cytoplasm of infected cells has been demonstrated both by staining technics (70) and chemical analysis (71) but this stage of the lesion occurs somewhat later and has been observed in other tissues during infections with unrelated viruses (72). The loss of potassium and creatin (14) secondary to muscle degeneration has been confirmed and it was shown that the excretion

of myoglobin in suckling mice is sufficiently large to cause a nephrosis (73) similar to that seen in the crush syndrome.

The acidophilic inclusion bodies observed in infected cardiac muscles (44) have been reported to occur in the liver, pancreatic acinar cells, skeletal muscle fibers, and fetal fat cells as well as heart muscle (74). The most likely interpretation was thought to be that they represent virus elementary bodies surrounded by an envelope of altered cytoplasmic material. This should be subject to confirmation in the electron microscope. To date, virus particles have not been identified in the lesions.

The remarkable events that take place in the pancreas following Group B infections have been beautifully illustrated in electron micrographs (75). Three changes were found. The earliest was the formation of cytoplasmic vacuoles which appeared focally after 18 hr. and enlarged and coalesced until the cytoplasm was filled with them. A second change consisted of concentrically laminated bodies that formed shortly after the appearance of the early stage of the vacuoles. These bodies were numerous 24 hr. following infection. And, finally, a few inclusions were seen which were at times as large as  $5\ \mu$  and quite dense. Internal structure was not defined. The phenomena of macrophage infiltration and fibrous replacement were illustrated as well as the sharp discrimination of virus action between the acinar cells and those of the islands of Langerhans.

Suckling ferrets have been added to the list of animals susceptible to the Coxsackie viruses (76). Characteristic lesions were induced by both Group A and Group B infections, including pancreatitis in the latter case.

It was shown by Rowe that adult mouse muscle is susceptible to infection with Group A strains if the leg were denervated at least seven days before inoculation. The agent was transmitted serially, using the inoculated muscle as source of the virus and injecting intramuscularly. Intraperitoneal inoculations sometimes succeeded if a large dose was used (77).

Coxsackie viruses, in general, are not readily adaptable to eggs or chick tissue culture. The chick embryo is susceptible to some strains of Coxsackie virus, Group A, Type 2, and characteristic lesions have been found in the muscles. Interestingly, the intrinsic muscles of the tongue were spared (78). In this little detail, too, the pathologic histology in the chick embryo resembles that in the suckling mouse. Shaw tested 29 strains in eggs, including three types of Group B and 10 of Group A. Only A 2 was unusually pathogenic for chick embryos (79). Godenne & Curnen (80) succeeded in cultivating the Easton-10, an A 8 strain, on the chorioallantoic membrane. Sixteen other strains failed to multiply. In flask culture of chick tissue the Group A, Type 4 strain, which Slater & Syverton had adapted to mouse tissue culture (81), grew, as did four Group A, Type 2 strains. Eleven other strains, including Types 1, 3, 4, 5, and 6 of Group A, failed to grow (79).

Purification of several Coxsackie viruses has permitted the measurement of individual particles (82). The diameter was determined to be  $37\ m\mu$  which is the same as that of poliomyelitis virus. These values are in good agree-



ment with measurements by ultrafiltration. Three Group A and two Group B types proved to be quite similar. All passed 34  $m\mu$  but not 24  $m\mu$  membranes (83). The Group A, Type 1 strain is said to be somewhat smaller (22).

#### SANITARY PRACTICES AND THE COXSACKIE VIRUSES

The three common disease-producing enteric viruses, infectious hepatitis, poliomyelitis, and the Coxsackie viruses, represent a challenge to sanitarians since proof is lacking that current sanitary methods regularly destroy them. Infectious hepatitis not infrequently appears in epidemic proportions following the contamination of water, and Swedish workers have observed an association between water contamination and poliomyelitis. Milk-borne outbreaks of poliomyelitis have been suspected. While all three infections presumably are as a rule transmitted rather directly and personal hygiene seems most important, it is desirable that the sanitary aspects of these diseases be kept in mind. Control of infection has proved to be the key to the suppression of other intestinal infections.

The Coxsackie and poliomyelitis viruses are relatively resistant to heat. Inactivation of mouse brain tissue suspensions requires approximately 55°C. for 30 min. (84). They are more stable if suspended in dairy products. While standard methods for the pasteurization of milk proved effective in destroying several strains of Coxsackie viruses, cream and, to a somewhat lesser degree, ice cream provided more protection, and high titered mouse passaged strains of virus were incompletely inactivated if suspended in cream and pasteurized at either 61.7°C. for 30 min. or 71.1°C. for 15 sec. (85).

Two studies of the effect of water and sewage treatment have recently been reported. The first was directed to the question of the reliability of the standard coliform plate count as a measure of the safety of waters. The survival of coliform organisms, a phage, a Coxsackie, and an encephalomyelitis virus (which causes a natural enteric infection of mice) were compared. It was found that the coliform plate count is a reasonably good yardstick although the Coxsackie virus was much more stable than the bacterium when stored in cold spring water (8 to 10°C.) and was more resistant to low concentrations of chlorine. The virus was more rapidly inactivated at room temperature (whether in water or sewage) and by ultraviolet light, suggesting that the coliform test is a conservative measure of the safety of swimming pools. The Coxsackie virus survived sewage treatment in a modern plant (86).

The destruction of pathogenic agents in water hinges on many factors, such as the absence of organic matter, time, and temperature. When these variables were controlled by the use of a purified virus preparation suspended in distilled water, the pH and temperature of which were likewise controlled, it was found that a Group A, Type 2 strain of Coxsackie virus required from 7 to 46 times as much free chlorine for inactivation as is needed to kill *Escherichia coli* (87). A virologist's view of the importance of sanitation in the enteric virus infections was expressed by Dempster (88).



The distribution of the various types of Cocksackie viruses varies considerably from year to year (89) and the occurrence of Group B strains has been intermittent. These patterns may be disclosed by the study of patients' or sewage specimens. Specimens collected during the past three years from a local sewage treatment plant, for example, have identified 1954 as a year of prevalent Group B strains quite as clearly as the clinical studies.

TABLE 1  
OCCURRENCE OF GROUP A AND B VIRUSES IN SEWAGE\*

Year	No. of specimens	No. that contained Cocksackie viruses	No. of Group A strains	No. of Group B strains
1952	56	31	35	1
1953	142	81	88	0
1954	152	74	55	20

\* From Kelly (90).

#### THE MUTUALITY OF COXSACKIE AND POLIOMYELITIS VIRUS INFECTIONS

Knowledge of the Cocksackie viruses provided proof that our late summer enteric infections are mixed and that both poliomyelitis and Cocksackie viruses play a part and thus inevitably raised the question of whether the two influence one another. There is now ample evidence that they do in experimental infections, whether of mice or tissue culture, and suggestive evidence, but no proof, that they do in man.

The first reported search for mutual effects was based on experiments in monkeys. A Group A virus was used and no evidence of interference noted (91). Later, a more thorough search was made using mice and chimpanzees as well as monkeys (92). Mixtures of poliomyelitis and Cocksackie viruses were titrated simultaneously in newborn and mature mice. The mixtures did not measurably influence the outcome. In other experiments, the mice were first inoculated with a Cocksackie virus and then challenged with Lansing poliomyelitis virus, the intervals selected being one hour, one or eighteen days. Subsequently, it has been learned that experiments of the latter type do indicate an interference if the intervals are appropriate and Group B strains are used (93, 94, 95). The Group A viruses have little or an opposite effect. The failure of the initial experiments may have been due to the choice of a Group A strain or the use of monkeys which are relatively resistant to infection.

It has now been repeatedly confirmed that several of the Group B viruses modify the course of experimental poliomyelitis in mice if the animals are infected several days prior to the inoculation of the poliomyelitis virus. The effect is shown by a prolongation of the incubation period in most animals and the suppression or mitigation of paralysis and death in some. Such ex-

periments are very rigorous for the mere inoculation of young mice adversely prejudices the outcome of experimental poliomyelitis much as the inoculation of unrelated substances does in man (96) and mice (97). Thus, the sparing effect of the Group B strains must overcome an opposite effect of the trauma and the usual experiment is weighted against a favorable outcome. This may account for the apparently accelerating effect noted when Group A strains are given as the first agent.

The interference of Group B infections on the course of poliomyelitis has recently been demonstrated to hold in tissue culture as well as in mice. Ledinko & Melnick (98) showed that the poliomyelitis viruses interfere with one another in tissue culture. Cells exposed to one strain and thereafter washed and exposed to another did not support the expected growth of the second. Several strains of Group A Coxsackie viruses tested under similar circumstances failed to interfere. (The strains used are all A 9.) Le Bouvier (99) confirmed the mutual interference of the poliomyelitis viruses in tissue culture using an experiment of somewhat different design and discovered that a Group B, Type 3 (100) Coxsackie virus gave the same effect. Thus, in tissue culture as in mice, the Group B strains alone have the ability to interfere.<sup>3</sup>

These observations have aroused interest in the possibility that similar phenomena occur in the field. In some cases the prevalence of Coxsackie virus infection can be inferred from laboratory tests of groups of patients. In others, satisfactory clinical evidence is available. Epidemic pleurodynia is sufficiently characteristic and striking clinically during epidemics as to leave little doubt of its nature and paralytic poliomyelitis is an equally satisfactory criterion of poliomyelitis virus infection. The etiologic nature of nonparalytic poliomyelitis is, of course, often open to doubt, particularly during outbreaks of Bornholm disease.

On the basis of these criteria, numerous investigators have observed that, during periods in which Coxsackie Group B virus infections have been prevalent, poliomyelitis has occurred infrequently or not at all (30, 32, 40, 102 to 109). Unfortunately, such data are not adaptable to statistical analysis and most authors would no doubt agree with Wilkins:

It is also of interest to note that while this widespread epidemic of Coxsackie Group-B virus infection was in progress, few cases of paralytic poliomyelitis were admitted to hospital. Similar observations have been made in Sweden and in the Union of South Africa. However, further investigation is necessary before it can be concluded that these two virus infections tend to be mutually exclusive—as the present evidence suggests (40).

Additional data bearing on the nature and extent of poliomyelitis during large, documented epidemics of Bornholm disease may be gleaned from older records. One of the biggest recorded outbreaks occurred in Sweden in 1931.

<sup>3</sup> One of Levaditi's last reports showed that a Group B Coxsackie virus suppressed the growth of poliomyelitis in mice when the two were passaged together (101).

Twelve thousand individuals were estimated to have been involved. The number of cases of poliomyelitis and of deaths due to poliomyelitis were simultaneously the lowest for the 20-year period and the absence of poliomyelitis was most striking, with one exception, in the particular provinces in which Bornholm disease was most common (110). It was this epidemic that crossed the Kattegat and afflicted the residents of Bornholm and prompted Sylvest's classical study (45). Epidemic pleurodynia reached such proportions in Denmark that it was made reportable for the years 1931-1937 (111). Two of these years were marked by large epidemics of pleurodynia, one by a very large epidemic of poliomyelitis. The data are again compatible with the theory of an inverse relationship not only for the country as a whole by years but for individual districts.

Epidemic pleurodynia was prevalent in Iceland and many parts of Europe during 1951. Reference has already been made (108) to observations of the absence or infrequency of paralytic poliomyelitis. In Great Britain outbreaks of pleurodynia were common that year (112, 113, 114) and the frequency of poliomyelitis was low (115). However, an unidentified virus infection may also have been prevalent during the summer of 1951 in Great Britain (116), the effects of which are wholly unknown.

In none of these instances are the data suitable to mathematical analysis. The substance of the evidence consists simply of the repetitious observation that the prevalence of Group B infections has been associated with unusually little poliomyelitis. This conclusion has been challenged on two occasions. Dekking (117) reported that Bornholm disease and poliomyelitis were both epidemic in Holland in 1951. The Dutch experience is, however, subject to an alternative interpretation. The available data consist of a rather comprehensive program of testing patient's feces for Coxsackie viruses during 1951 and the official monthly reported cases of poliomyelitis (118). During the May-August period, Group B strains were isolated from 27 of 109 fecal specimens (119, 120). This in our experience would indicate a high level of infection. A few specimens collected earlier in the year had yielded no viruses whatever. Of 174 collected after September 1, only six were infectious and these yielded only Group A strains. Thus, there is evidence that Group B Coxsackie virus infections were prevalent but only during the May-August period and it may be significant that the peak incidence of poliomyelitis was not reached until October, two months later. The discrepancy in time was noted by van Creveld (121) who wrote: "The epidemic of Bornholm disease occurred in a period when there were practically no cases of poliomyelitis." The peak incidence of poliomyelitis in Holland for the 1942-48 period, as well as for 1952, fell in August. The dislocation in 1951 was therefore unusual.

A similar course of events was noted by Vivell in Southern Germany where Bornholm disease was also prevalent during the same summer and frank cases of poliomyelitis did not occur until late fall (122).

The Dutch experience resembles our own in 1954 in upstate New York where Group B infections, as revealed by the rate of isolation from fecal

specimens from patients and from a methodical sampling of sewage, were found to be limited to the period ending in early September. Poliomyelitis was infrequent in the upstate area during the year, especially infrequent during the period in which the Group B viruses were present. The peak incidence occurred in mid-September with a second lower peak early in October. It is noteworthy that 1948 and 1954, the two years in which Group B strains have been proven to be prevalent in New York, were both years in which little paralytic poliomyelitis occurred in the upstate area.

The second expression of a contrary opinion is in a report by Melnick (123) who observed that a North Carolina epidemic (26) was marked by the severity of the poliomyelitis and the large number of Group B strains isolated. Of the 19 strains of Coxsackie virus isolated 16 were members of Group B. The isolations were made from flies, sewage specimens, and, in two cases, pools of fecal specimens. Whether the presence of the Group B viruses corresponded in time and place with the poliomyelitis epidemic cannot be determined from the report. Many outbreaks of both diseases are at times quite circumscribed. For example, a survey in Texas during 1948 also yielded evidence of the presence of both Group B viruses and poliomyelitis (124). A table reveals that the poliomyelitis was concentrated in two counties while the isolations of the Group B viruses were more numerous in two others, in those in which poliomyelitis was infrequent.

Counties	Cases of poliomyelitis	Number of Group B isolations
Alamo Donna	2	5
Pharr McAllen	35	1

The probability of such a distribution occurring by chance  $\cong 0.000125$  but the probability that the presence of the Group B strains was responsible cannot be calculated. However, it seems clear that field data must be analyzed in terms of time and place for the influence of an interference, if any, can hardly be expected to extend to areas in which the interfering agent is not present or to persist after its disappearance.

Attention should be directed to the simultaneous occurrence of Group B Coxsackie and poliomyelitis viruses in carriers and patients for an interference, if it occurs in man, might be most apparent in the enteric phase of the disease where it could lead to a depression of infectivity and carrier rates. This would fit our observation that years of high Bornholm and low poliomyelitis incidence have frequently been followed by years of epidemic poliomyelitis which might be expected if subclinical infection and immunization had been suppressed by the Group B infections. With the newer tech-

nics it may prove possible to investigate more thoroughly whatever interrelationships exist among the enteric viruses. Much has been learned about the antagonisms between certain enteric bacteria (125) and there is no reason to doubt that biologic laws apply to viruses as well as bacteria.

The relative sensitivity of different technics and the possibility of interferences in the tests masking the occurrence of a second virus must all be explored. Meanwhile, it may be of some interest to note the distribution of the two kinds of Cocksackie viruses in fecal specimens collected from paralytic and nonparalytic cases of poliomyelitis. The records of 484 specimens are available (1, 2, 117, 126 to 137). Of these 282 represent cases of paralytic and 202 nonparalytic disease. In the two groups 54 and 51 isolations of Cocksackie viruses were made. It may be significant that the paralytic cases yielded only Group A strains while the nonparalytic yielded 22 Group A and 29 Group B viruses. The findings in the latter group are not surprising since, in the light of current knowledge, it is possible that many of them were cases of Group B Cocksackie virus infection rather than poliomyelitis. Only 16 were proved to be infected with a poliomyelitis virus by isolation of the agent. On the other hand, this does not explain the absence of Group B strains from the cases of paralytic poliomyelitis. Data of this kind may be useful in testing the hypothesis that a mutually exclusive relationship exists in human infections. As far as the author can determine, poliomyelitis and Group B Cocksackie viruses have rarely been isolated from individual fecal specimens although poliomyelitis and Group A strains have often been associated. This, too, deserves further attention.

The 1954 Annual Report of the Queensland Institute for Medical Research (138) includes a report of a mixed epidemic of influenza B and Cocksackie B 4 virus which seemed to spread throughout the country together. Both agents were identified on several occasions in different parts of Australia.

#### NOMENCLATURE—REVIEWS

In revising Holmes's classification of animal viruses, Van Rooyen proposed that the Cocksackie group be considered a Family of a single genus and two subspecies corresponding to Groups A and B (139). European workers frequently refer to the Cocksackie viruses as strains of Pseudopoliomyelitis. This terminology was endorsed by the Third European Conference on Poliomyelitis and by Mollaret in a detailed discussion of the problem of nomenclature (140). It was also used by Keller & Vivell (137) who adopted Jungelblut's proposal (141) of a family of poliomyelitis viruses that would include the classical Poliomyelitis viruses (Types I, II, and III), Parapoliomyelitis virus (the EMC, Columbia SK, MM, Mengo strains), and Pseudopoliomyelitis viruses (the Cocksackie family). Another suggestion has been that the latter be designated as a Genus Cocksackie, Tribe Parvovirae of a Family of Enteroviraceae (142). Zhdanov designated the Cocksackie viruses as Genus Myophilus within the Family Polyvetaeaceae and the Order Arthropodo-

philiales (143). The latter seems quite incongruous. The term Pseudopolio-myelitis has been justified by similarities among the viruses, the experimental lesions, and the clinical nature of certain cases of Coxsackie virus infections but is an inappropriate name for epidemic pleurodynia, herpangina, and infantile myocarditis, or the agents that cause these dissimilar diseases.

Early studies of the Coxsackie viruses were discussed in a previous volume of the *Annual Review of Microbiology* (144). A number of extensive reviews have since been published, including that of Keller & Vivell (137) and monographs by de Goës (145), Delpy (146), Finger (147), and Pohjanpelto (148).

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## FUNGUS INFECTION OF PLANTS<sup>1,2</sup>

BY CARL J. EIDE

*Department of Plant Pathology and Botany, University of Minnesota,  
Institute of Agriculture, St. Paul, Minnesota*

Gaumann (1) defines infection as that phase of infectious disease which extends from the germination of the pathogen to the time of its entry into a stable parasitic relationship with its host. It is fairly easy to follow the behavior of a fungal germ tube after germination and consequently to distinguish between germination and the early phases of infection. It is not so simple to determine when the fungus enters into a "stable parasitic relation" with the plant, especially since some fungi do not become parasitic in the sense of obtaining nourishment from living host cells, but instead live as saprophytes on tissue killed in advance of their own hyphae.

In his review of the physiology of host-parasite relations, Brown (2) divides the processes involved into three stages: before penetration, penetration, and after penetration. The present review attempts to cover recent literature on these stages, with some limitations. Spore germination as such will not be covered, except as it touches infection in some special or unique way; and the processes after penetration will be followed only to the point at which success or failure in establishment of parasitic relations seems assured.

### THE STIMULI TO PENETRATION

Fungi, germinating as they usually do on the surface of the plant, often have characteristic and rather elaborate means for penetration, exhibiting an active response to stimuli. The stimuli that cause fungal germ tubes to grow in certain directions, and to penetrate stomata, wounds, or unbroken plant surfaces were reviewed by Brown (2). Recently, Dickinson (3) found that the germ tubes of 11 species of rusts and six powdery mildew fungi grew upright into the air when the spores germinated on water or on 2 per cent gelatin in petri dishes. The behavior of the germ tubes from spores germinated on collodion membranes, which were in contact with water below but visibly dry above, depended upon the preparation of the membranes and their subsequent treatment. One such factor was the period during which the solvent was allowed to evaporate before the membranes were put into water. More germ tubes grew into the air on membranes dried for 30 sec. than on ones dried for 90 sec. More upright germ tubes were formed on membranes soaked in 95 per cent alcohol for 24 hr. than on membranes soaked in water or lower percentages of alcohol for the same length of time. The percentage of

<sup>1</sup> The survey of literature pertaining to this review was completed in 1954.

<sup>2</sup> Paper No. 882 of the Miscellaneous Journal Series of the Minnesota Agricultural Experiment Station.

upright germ tubes was also greater when the relative humidity above the spores was high.

Dickinson concludes that the stimuli affecting the germ tubes in his experiments were positive hydrotropism (growth toward water or moisture), negative thigmotropism (growth away from a solid surface), and dia-thigmotropism (growth along a surface). He believes that the thigmotropic responses are profoundly influenced by the nature of the surfaces, and that the membranes in host plant cells are of fundamental importance in determining the parasitic behavior of obligate parasites.

Thigmotropism in *Puccinia graminis tritici* was described by Johnson (4) as resulting in germ tube growth at right angles to the long axis of the wheat leaf. This response might have some survival value, since the germ tube would have a better chance of contacting a stoma than it would if growing parallel to the leaf.

Germ tubes of *Peronospora schachtii*, *P. parasitica*, *Cladosporium fulvum*, *Verticillium dahliae*, *Botrytis cinerea*, and *Monilia fructigena* reacted to membranes much as did germ tubes of rusts and powdery mildews [Dickinson (3)]; i.e., some grew upright, depending upon the membrane on which they germinated. None of the germ tubes of any of the fungi listed above grew upright on water or 2 per cent gelatin; in contrast, all of the rusts and powdery mildews studied produced upright germ tubes on these media. None of the germ tubes of *Ustilago violaceae* or *Aspergillus niger* grew upright under any of Dickinson's experimental conditions.

In a later paper Dickinson (5) described the development of *Puccinia triticina*, *P. graminis*, and *P. glumarum* on artificial membranes prepared from 0.05 per cent paraffin, 0.4 per cent collodion in ether-alcohol, and cell fragments from leaves of susceptible wheat varieties. On such membranes he found structures corresponding in morphology and color to the appressoria, substomatal vesicles, and infection hyphae produced normally by these three rusts in infected plants. The "appressoria" adhered to the membrane, and, if the membrane were thin, a stalk from the "appressorium" penetrated it and produced a "substomatal vesicle" on the other side. The "infection hyphae" frequently ended in swellings that might have corresponded to haustoria. The "substomatal vesicles" developed on membranes made with wax congealing at 52° C., but not on ones made with wax congealing at 36° C.

Dickinson (6) also studied the behavior of germ tubes of *Erysiphe graminis* on several types of membranes. This fungus normally penetrates host epidermal cells without forming substomatal vesicles and infection hyphae. On membranes prepared from collodion and wax congealing at 52° C., appressoria were formed by germ tubes and evidence of attempted penetration was found. Penetration was successful, but atypical, on very thin membranes. On double membranes (paraffin wax over gelatin) the wax layer was penetrated by a stalk similar in diameter to that formed on a living host but shorter.

Further evidence of the effect of contact stimuli is reported by Schwing-

hamer (7) for *Colletotrichum linicolum*, which causes anthracnose of flax. Conidia of this fungus germinate and form appressoria on glass and other surfaces such as cellophane. The latter substrate is penetrated by an infection peg very similar to that found on the host plant.

Evidence of both contact and chemical stimuli was found by Van Burgh (8). She germinated spores of *Colletotrichum phomoides* on formolized gelatin membranes of different degrees of hardness, and found no appressoria formed on the softest, which was most quickly penetrated by the fungus. The number of appressoria formed increases with the hardness of the membrane. On the other hand, no typical appressoria were formed on any membranes if nutrients were present in the infection drops. A similar tendency was observed in the germination of spores of *C. linicolum* by Schwinghamer (7).

Paddock (9) states that the growth of the germ tubes of conidia of *Helminthosporium victoriae* on leaves of susceptible oat varieties is primarily haphazard, although the appressoria were formed most readily at the junctures of cells surrounding the stomata and at the junctures of epidermal cell walls. Since slight irregularities occur at these places, Paddock considers the formation of appressoria to result from thigmotropic responses.

These results seem to indicate that the stimuli to penetration are frequently not highly specialized. Furthermore, earlier workers (10 to 14) found that resistant varieties of plants were penetrated as easily by certain fungi (mostly rusts) as were susceptible ones except when morphological barriers existed, again indicating an absence of special stimuli to penetration in susceptible hosts. Müller (15) recently found that *Phytophthora infestans* (potato late blight) penetrates such hosts as *Lactuca sativa*, *Dahlia* var., and *Phaseolus multiflorus*. The usual host range of this fungus includes only members of the Solanaceae, principally potato and tomato. Pristou & Gallegly (16) observed that the process of penetration by *P. infestans* was the same on susceptible and resistant potato varieties. On the other hand, Paddock (9) found that different varieties of oats differed in their penetrability by germ tubes of *Helminthosporium victoriae*. This effect, which was apparently chemical, will be considered in more detail later.

The sites of fungal penetration are fairly well fixed for obligate parasites, e.g., germ tubes of rust sporidia usually penetrate the unbroken cuticle, while urediospore germ tubes go through stomata. Recently Schwinghamer (7) found a similar specificity in *C. linicolum*, which penetrates, by means of appressoria, only through the cuticle. Other fungi show more diversity in penetration. For example, Pristou & Gallegly (16) found that zoospore germ tubes of *P. infestans* usually penetrate the cuticle, but that occasional stomata are penetrated. In either case an appressorium is always formed, a fact that was missed by some earlier workers. Pristou & Gallegly were unable to find penetration by germ tubes from sporangia, but such penetration was observed by Ferris & Kent (17). *H. victoriae* penetrates susceptible oat varieties through the cuticle or stomata, with or without the aid of appressoria (9). Seventy per cent of the penetrations were through the

cuticle with appressoria. Direct penetration without appressoria was observed only on the variety Vicland (susceptible), and then only where a trichome or a juncture of cells formed a barrier against which the germ tube lodged. At such sites the cuticle seemed to be ruptured mechanically.

Paddock (9) states that there was no evidence of a mucilaginous sheath to hold the appressorium of *H. victoriae* to the leaf, and Dickinson (5) also failed to demonstrate such a coat on the appressoria of *P. triticina*.

#### FACTORS AFFECTING PENETRATION AND INFECTION

The penetration of plants by fungi is influenced by other host factors, such as chemical and morphological barriers. Earlier literature on this subject has been reviewed by Hart (18) and since then few other findings have been published. Sörge (19) found that a variety of peas resistant to foot rots produced a toxic substance in the testa. Decoctions of the testa reduced and retarded mycelial growth and production of pycnidia by *Ascochyta pisi*, *A. pinodella*, and *Mycosphaerella pinodes*, which cause the foot rot. Hafiz (20) found that certain varieties of gram (*Cicer arietinum*) were resistant to blight partly because they had more glandular hairs on the stems and leaves than did susceptible varieties. The hairs secreted malic acid, which in high concentrations inhibited spore germination and retarded growth of the pathogen, *Mycosphaerella tritici*. Differences in the toxicity of water from the foliage of resistant and susceptible varieties were apparent only if the plants were 40 days or more old. Differences in susceptibility to infection due to age were found also by Carpenter (21) in the Hevea rubber tree. Leaves 1 to 4 days old were highly susceptible to infection by *Pellicularia filamentosa* (target spot). In leaves 4 to 7 days old the percentage of infection decreased rapidly, the period of greatest susceptibility being before that of greatest leaf expansion.

Cohen (22) showed that the manner of penetration by certain members of the order Peronosporales was dependent upon host variety and environment, as well as upon the species of fungus. Percentages of penetration through stomata by *Bremia lactucae* on lettuce varied from 0 to 100 per cent, depending upon the incubation temperature and the age and morphology of the leaves. There was a higher percentage of stomatal penetration on Romaine lettuce than on Bibb lettuce, and on the upper than on the lower leaf surface.

Recently Bald (23) has offered evidence that stomatal guttation is an important factor in penetration of stomata by fungi. This author found very tiny droplets of water over the stomata of gladiolus plants subjected to low temperatures, high atmospheric humidities, and high soil moisture. On exposure to dry air the droplets were drawn into the stomata. Germ tubes of *Pleospora* spp. ascospores were observed to have entered stomata after the retraction of the droplets. If a film of water lay over the surface of the leaf, no penetration occurred, although the hyphae grew extensively over the surface



of the leaf. When this film dried and the plants were placed where stomatal guttation occurred, many of the hyphae penetrated stomata. This might be interpreted as an instance of hydrotropism, but conceivably the retraction of the droplets might mechanically direct the growth of the hyphae into the stomata.

Bald found also that if gladiolus leaves were kept wet, *Botrytis gladiolorum* penetrated the cuticle directly. When the leaves were alternately wetted and dried, many germ tubes penetrated stomata. Bald suggests that this phenomenon may account for epidemics of such diseases as rust caused by *Puccinia antirrhini* in arid areas, and that direct penetration at the junctions of epidermal cells may result from imbibition of water by pectin in intercellular spaces.

Yarwood (24) found that leaves of young beans (*Phaseolus vulgaris*) floated on water in a humid chamber, produced guttation water on the surface exposed to the air. When the leaves were inoculated with spores of *Uromyces phaseoli* or *Colletotrichum lindemuthianum*, the resulting infection was positively correlated with the amount of guttation. The same correlation was observed if leaves of broad bean were inoculated with *Botrytis cinerea*.

The findings of Bald (23) and Yarwood (24) are in some respects similar to those of Johnson (25, 26, 27), who had earlier investigated effect of water congestion of plants on their susceptibility to fungal pathogens. Johnson worked on bean anthracnose (*C. lindemuthianum*), late blight of potato and tomato (*P. infestans*), leaf rust of oats (*Puccinia coronata*), wheat stem rust (*P. graminis tritici*), sunflower rust (*P. helianthi*), and corn rust (*P. sorghi*). The host plants of these pathogens became spontaneously water-congested (i.e., the intercellular spaces of the leaves became filled with liquid) following certain types of treatments, and the ease with which it occurred depended upon the species and variety. Water congestion in potted plants could be induced most uniformly by placing the plants out of doors for five to ten days, followed by periods of up to 24 hr. in a special moist chamber in the greenhouse. In some species and varieties the water congestion was apparent to the unaided eye as "water-soaked" spots; in others it could be seen only by the use of a microscope, or by placing the leaves in a harmless dye.

In most of Johnson's experiments the water-congested plants "were more predisposed to infection than uncongested plants of the same variety" (27). He indicates degree of infection by symbols, and it is not always clear whether he refers to the number of infections or the severity of their subsequent development (26). In a number of instances, e.g., anthracnose of bean, crown rust of oats, and late blight of potatoes, it is clear that a greater number of infections occurred on the congested plants. He points out that although congested Vicland oats (resistant to rust) had more infections than State's Pride (susceptible), Vicland was resistant to the subsequent progress

of the fungus. This was undoubtedly due to the specific physiological resistance (hypersensitivity) of the Vicland variety, although the race of *P. coronata* was not identified. Johnson says that it is fairly certain that little or no infection by leaf rust occurred in the absence of water congestion.

Cohen (28) induced water congestion of bean leaves in a vacuum desiccator and found that it did not interfere with the formation of appressoria, with stomatal penetration, or with the formation of sub-stomatal vesicles by *Uromyces phaseoli*. He does not say whether these processes were increased by water congestion, but the inference is that they were not. He does emphasize that the development of infection hyphae from the sub-stomatal vesicles was rare on the congested leaves, and that the result was fewer pustules. If the leaves were allowed to recover from the water congested condition before inoculation, the number of pustules was greater than on the noncongested controls.

Yarwood (29) suggests that the effects of pressure on infection which he observed might be due to consequent water congestion. Bean leaves pressed between two rubber corks at pressures up to 170 lbs. p.s.i. showed fewer pustules of *U. phaseoli* than controls, if the treatment was applied either immediately before inoculation, or during the following 48 hr. On the other hand, the pressure treatment had no protective effect if applied more than an hour before inoculation. Water soaking the leaves by spraying the lower surfaces also prevented the development of pustules from young infections, but was less effective than the pressure treatment. Factors other than water congestion might have been involved. Pressure after inoculation also reduced infection by *Erysiphe polygoni* on bean, by snapdragon rust (*P. antirrhini*), by sunflower rust (*P. helianthi*), and by spinach downy mildew (*Peronospora effusa*). Water soaking leaves 15 hr. after inoculation reduced the number of lesions caused by spinach downy mildew as much as pressure did. Pressing bean leaves 10 or more days old increased their susceptibility to anthracnose (*C. lindemuthianum*).

White & Baker (30) found that when barley leaves were exposed to pressures of 5 to 30 lbs. p.s.i. just before inoculation with *E. graminis hordei*, there was decreased infection at the higher pressures. They suggest that the effects observed by them and by Yarwood (29), were caused by the release from injured or killed host cells of substances which affected the pathogen like the substances released from parasitized hypersensitive cells, to be discussed later in this review. It seems possible that water congestion caused by spraying leaves or by subjecting them to a vacuum, as in the experiments of Cohen (28), might injure some of the cells and lead to similar results.

#### PENETRATION OF PLANTS THROUGH WOUNDS

So far this review has dealt principally with fungi that penetrate cuticle, stomata, or other natural openings. Some of these fungi may also invade wounds in the plant, but there is a large group of fungal pathogens that can

penetrate only through wounds, and hence depend upon other agencies to break through the plants' protective coverings. These agencies are usually primary factors in determining whether or not such fungi are able to penetrate and parasitize a plant. No attempt is made here to record all the instances of wound penetration which have appeared in recent literature; the following investigations were selected because they illustrate new or unusual types of injuries, or new situations leading to wound penetration by fungi. The penetration and establishment of heart-rotting fungi in living trees has been well covered in a recent review (31).

An accumulation of salts in guttation droplets on the leaf-tips of stock (*Matthiola incana*) resulted in small necrotic areas through which *Botrytis cinerea* could infect, according to Baker, Matkin, & Davis (32). This type of infection could be largely prevented by fungicidal sprays; but when the sprays were applied during the blossom period, the flowers were injured, enabling the fungus to infect the blossoms. *B. cinerea* is able to penetrate the uninjured petals of some plants, but not those of stock. It frequently penetrates through injured or moribund parts, e.g., on greenhouse cucumber (33). Infection of dead blossoms, tendrils, etc., leads to invasion of sound tissue, resulting in the girdling of the stem at the nodes.

Changes in agricultural practices sometimes result in injuries that permit the entrance of pathogens. Cunningham (34) reports that dry rot of potatoes by *Fusarium* spp. in storage bins is increased by the use of sprout inhibitors. If these materials are added as dusts, the rot is most abundant where the tubers drop from the conveyor and an excess of dust accumulates. Histological studies showed that sprout inhibitors containing the methyl ester of naphthalene acetic acid delayed suberization of cut surfaces and partly or entirely prevented the formation of wound cork. Materials containing tetrachloronitrobenzene caused irregular suberization and delayed the formation of wound cork. *Fusarium sambucinum* penetrated tubers if the wound cork layer was absent or irregular, but was effectively stopped where it was complete. None of the inhibitors affected in any way the growth of the fungus.

Commercial methods of handling potatoes which result in bruising increase the incidence of dry rot caused by *Fusarium*, as emphasized recently by Foister & Wilson (35). In Britain, most dry rot infection follows grading in mechanical reciprocating riddles, where the potatoes are damaged by the bare wire screens. Boyd (36) showed that certain potato varieties were less liable to infection through riddle injuries because of their greater relative resistance to abrasion. When inoculated by injection, such varieties might be highly susceptible. In the United States, better insect control by use of DDT and the adoption of later-maturing varieties during the past 10 years has increased the harvesting of immature potatoes. Combined with mechanical harvesting and bulk handling from the field to the bin, this has increased the bruising which permits the entrance of dry-rot fungi, especially where conditions do not favor the rapid healing of such wounds.

For short periods after leaf fall, leaf scars expose tissue often highly susceptible to fungus invasion (37, 38). Crowdy (37) observed that the conidia of *Nectria galligena* are literally sucked into tracheids exposed by leaf fall on apple twigs. The suction could be demonstrated by placing dye solutions on the leaf scars; it started to diminish 5 to 15 min. after the leaf was detached, and could no longer be detected after 2 to 3 hr. The spores were sucked only a short distance into the tracheids, but were able to germinate and become established in the protective environment thus provided. Further development was dependent upon the ability of the host to form a protective barrier around the incipient lesion. Infection of camellias by *Glomerella cingulata* also occurs principally through leaf scars. Baxter & Plakidas (38) also found that the fungus penetrated the leaf scars more slowly as the time of inoculation was increased up to 48 hr. after leaf fall.

Avenues of entrance for certain fungi are often opened by the attacks of other pathogens. Recently Schultz (39) reported that potato tubers of the Kennebec variety, which is resistant to late blight, are invaded by *Phytophthora infestans* through the lesions of powdery scab (*Spongospora subterranea*). Approximately 30 per cent of the tubers with scab were infected with blight, but the scab-free tubers were not. The lesions of both powdery scab and late blight provide entrance for *Fusarium caeruleum* (35). Although the lesions caused by these fungi and wounds made in handling potatoes frequently permit the entrance of dry rot fusaria, growth cracks and damage caused by wire worms or slugs do not (35). Snapdragon rust (*Puccinia antirrhini*) was found by Dimock & Baker (40) to permit the entrance of facultative parasites, principally *Fusarium* spp. In humid climates these fungi caused more damage to the snapdragon than the rust did. According to Ou & Walker (41) isolations from anthracnose lesions on pea stems usually yielded not only the anthracnose fungus, *Colletotrichum pisi*, but also *Ascochyta* spp. When pea plants were inoculated with *C. pisi* alone, the spores germinated and produced appressoria in what appeared to be a normal manner, but only a few penetrated the leaves and none the stems. *C. pisi* was found also to be an unaggressive parasite when inoculated into the stem through wounds. Double inoculation with *C. pisi* and *Mycosphaerella pinodes* (perfect stage of *Aschochyta* spp.) resulted in lesions from which both organisms could be isolated. The authors cite a number of other examples of species of *Colletotrichum* which infect only through wounds or following other parasites.

Occasionally infection of plants by fungi is inhibited by the presence of other organisms or viruses. Infection of potato foliage by *P. infestans* is reduced if the plants are infected with viruses X or Y [Müller & Munro (42)]. These authors found that the actual number of infections is smaller on virus-infected plants, and that fructification on individual lesions is also reduced. They conclude that the mechanical structure of the epidermal cells may be altered by the virus, increasing the difficulty of penetration, but that physi-

ological changes in the plant, affecting the nutrition of the fungus, are also evident. Infection of sweet potato sprouts with *Fusarium solani* f. *batatas*, which causes a limited basal rot of the sprouts, prevents subsequent infection by *F. oxysporum* f. *batatas*, according to Bega (43). The latter organism causes a wilt, and both are wound parasites.

There are numerous instances of the penetration of fungi into plants through wounds made by insects. Christensen & Schneider (44) point out that the European corn borer (*Pyrausta nubilalis*) not only provides a means of entrance for fungi causing shank, stalk, and ear rot of corn, but also assists in the establishment of some of these fungi in the tissue. They found that the frass left in the tunnels by the borer is an excellent medium for the growth of fungi. Some, e.g., *Penicillium oxalicum*, are weak parasites, but if able to grow on dead material, kill the tissues in advance and thus establish a parasitic relationship with the host. A similar situation exists in the relationship of the raspberry cane midge, *Thomasiniana theobaldi*, to three fungi found on raspberry canes (45, 46). This insect lays eggs in naturally occurring splits in the outer cortex of the cane. The larvae feed on tissues exposed by the split and damage the periderm so that it becomes a suitable substrate for *Leptosphaeria coniothyrium*, *Didymella applanata*, and *Fusarium culmorum*. The first two are parasites causing cane blight and spur blight, respectively, of raspberry in the absence of the midge. However, *D. applanata*, which is normally confined to the outer cortex of the cane, is able to penetrate to the pith through the midge injuries, and so causes much greater damage. *F. culmorum*, a parasite of cereals, is known to attack the raspberry only when this plant is injured by the midge. It may also penetrate to the pith. The midge is not a vector of this fungus, and splits in the cane not infested by the midge are not susceptible to infection by this fungus.

Seeds are often the point of attack of fungal parasites, and injuries or natural weaknesses of the seeds of certain species or varieties may provide means of penetration. Christensen, Olafson, & Geddes (47) found that storage molds are common in cotton seeds, despite their heavy seed coat. The vulnerable point in this armor is the hilum, protected only by a plug of thin-walled parenchyma cells, which shrink in a mature seed. When the seed is stored at high relative humidities, storage molds are found first under the seed coat near this pore.

Harvesting, threshing, and other operations, as well as inherited defects, are of great importance in determining the penetration of agricultural seeds by fungi, including both plant pathogens and storage molds. Moore & Christensen (48) found that threshing dry flax resulted in considerable injury to the seed coats, many of the injuries being too small to detect with the unaided eye; when these seeds are planted, a number of soil fungi enter the cracks, and poor stands result. Chemical protectants resulted in large stand increases when used on such damaged seed.

In some flax varieties, especially the yellow-seeded ones, the seed coat

splits at the small end of the seed, often while it is still in the boll (48). This makes such varieties especially vulnerable to soil-borne fungi. According to Kommedahl & Culbertson (49), who studied paired isogenic lines of yellow and brown-seeded flax varieties, the yellow-seeded lines contained 6 to 15 times more split seed coats. Brown-seeded lines produced stands 1.3 to 2.6 times those of the yellow-seeded ones.

A similar susceptibility to autogenous injury in corn was ascribed by Hoppe (50) to genetic factors. Breaks in the pericarp of the crown of the seed were observed in one hybrid, while another in an adjacent field was free from the injury. These breaks were practically all infected with a species of *Hormodendrum* that sometimes destroyed the entire kernel. More common wound parasites, such as *Fusarium moniliforme* and *Penicillium* spp., were absent.

Special cases of secondary infection of plants by fungi are caused by the ability of spores of certain fungi to move through the vascular systems of plants. Such plants are usually infected in the strict sense of the term at one or more places, and from these points the rapid mechanical spread of conidia through the vessels may result in numerous additional infections where the spores lodge and germinate. According to Banfield (51), this explains the rapid invasion of the elm by *Ceratostomella ulmi* in the season following initial infection. The fungus, after becoming established, produces numerous yeast-like spores that are carried throughout the tree by the sap stream in the large vessels of the new annual rings. Banfield demonstrated that these spores would move in a few hours from the base nearly to the top of a tree 35 feet tall. The conidia of *Chalara quercina* were found to be generally distributed in the xylem vessels of oak trees with the oak wilt disease [Young (52)]. Because the vessels of white oak are smaller and more occluded with tyloses than those of red oak, Young thinks that the slower invasion of the former might be due to slower movement of the spores through the vessels. A similar type of resistance is reported by Edgerton (53) in sugar cane. Conidia of *Phylospora tucumanensis* (red rot) move through the vessels of the stalks and leaf mid ribs of sugar cane, producing numerous lesions along the vessels. In some varieties of cane the cross walls in the vessels remain intact at the nodes, which makes such varieties resistant to the invasion of the fungus.

Scheffer & Walker (54) placed tomato cuttings in suspensions of microconidia of *Fusarium oxysporum* f. *lycopersici* for several hours, and, at the end of that time, were able to isolate the fungus as far as six inches above the cut end. Spores of the pathogens, as well as of non-pathogenic fusaria, moved with equal ease in the vessels of resistant and susceptible varieties (55). These authors did not demonstrate that conidia were present in naturally infected tomato plants.

#### INFECTION PROCESSES AFTER PENETRATION

Penetration of a plant does not complete the process of infection if we

accept Gaumann's (1) concept of infection. Subsequent behavior of a fungus leading to its establishment as a parasite may be simple or complex, and in many cases is not well-known.

Sometimes the pathogen becomes quiescent after penetration and cannot become parasitic until the resistance of the host is lowered in some way (56, 57, 58, 59). For example, Rowell (56) found that *Alternaria dauci* f. *solani* penetrated young tomato leaves, but produced no symptoms unless the plants were shaded or otherwise predisposed. He was able to isolate the fungus from the symptomless young leaves. Older leaves were infected and symptoms appeared without shading. *Alternaria tenuis*, which causes a leaf blackening of sugar beets, is also unable to parasitize young, vigorous leaves (57).

Christensen (58) found that *Cytospora chrysosperma* is an apparently normal inhabitant of the bark of healthy poplar, willow, and mountain ash trees. Apparently healthy pieces of branches or twigs of these trees, when surface-sterilized and covered with paraffin, produced abundant fruiting bodies of the fungus. The time and manner of infection is unknown. On the other hand, inoculation with the fungus through wounds in vigorous trees rarely resulted in cankers.

Similarly, sugar cane was found to be infected in the nodal region with *Phylospora tucumanensis* (red rot), but the rot did not develop unless the canes used as seed pieces were planted, or unless the canes were held at 70°F. for 20 to 30 days [Steib & Chilton, (59).] This fungus apparently infects through the leaf scar, bud, and root band tissues while these structures are covered by the leaf sheath. Dipping seed canes in conidial suspensions did not increase the incidence of disease, and even inoculation of the growing stalk, if the leaves were removed, was relatively ineffective.

Fall (60) found that strawberry leaves could be penetrated by *Mycosphaerella fragariae* at 15°C., but no lesions were produced at that temperature. Symptoms appeared at 25°C.

*Post-penetration stimuli.*—The cytology of the relationships between parasitic fungi and their hosts has been reviewed by Rice (61, 62) who emphasized investigations of the rust fungi and considered in detail the facts and speculations about the nutrition of these fungi. Dickinson (5) continued his investigations of the growth of rusts on artificial membranes in an attempt to find out more about the formation of haustoria and other structures produced by the rusts. As stated previously, he found that structures similar to appressoria, substomatal vesicles, and infection hyphae were formed by the germ tubes of urediospores of several cereal rusts when the spores were germinated on membranes which contained wax congealing at 52°C. When such membranes were placed over the exposed mesophyll of susceptible wheat leaves, some of the infection hyphae from the "vesicle" beneath the membrane formed haustoria and penetrated the mesophyll. If the wax used in the membrane congealed at 36°C., the urediospores germinated, but no appressoria or vesicles were formed, and, if the germ tubes



contacted the host mesophyll directly, no signs of haustorial formation were found. Penetration of stomata and the formation of appressoria and vesicles seem prerequisite to haustorial formation and the penetration of the host cells.

In later work Dickinson (63) found that mycelium of *Puccinia triticina* and *P. graminis* would grow out in tufts from infected wheat leaves stripped of the epidermis. Such hyphae of *P. triticina* were brought into contact with the collodion-wax membranes by placing the stripped mesophyll on the membrane. If the membrane contained a wax congealing at 52°C. or higher, it was penetrated and a haustorium-like structure protruded on the opposite side. Again the stimulus seemed to be one of contact, although, since it occurred only on membranes containing paraffin wax with a relatively high congealing point, the possibility of a chemical stimulus was not excluded. Similar behavior by *Erysiphe graminis* was observed when the spores were germinated on a double membrane of paraffin wax over gelatin (6). Here the wax layer was penetrated and the gelatin invaginated by a haustorium-like structure which differed from that found in plant cells by the absence of the usual finger-like processes.

These studies led Dickinson to conclude that a series of contact stimuli were responsible for the development of the obligate parasites in question on and in the hosts, and that such stimuli could be simulated to some extent by artificial membranes. He considered the same or similar stimuli to be responsible for associated phenomena such as nuclear division in the rust hyphae. However, he does not exclude the possibility of chemical stimuli.

Chemical stimuli are also important in the development of rust fungi (64, 65). Hurd-Karrer & Rodenhiser (64) observed structures produced by germinating urediospores of *Puccinia graminis tritici*, *P. triticina*, *P. dispersa*, *P. coronata*, and *P. sorghi* which resembled morphologically the appressoria, substomatal vesicles, and infection hyphae formed by these rusts in susceptible hosts. These structures were formed on about 0.1 per cent of the germ tubes placed on an agar medium containing glucose and mineral salts, but were not formed on water agar. There was no apparent evidence of contact stimuli.

Sharp & Smith (65) observed that as many as 40 per cent of the germinating urediospores of *P. coronata avenae* produced appressoria, substomatal vesicles, and short infection hyphae on an artificial medium. The medium was 3 per cent gelatin, adjusted to pH 6.2-6.6, and containing ZnSO<sub>4</sub>. Vesicle formation was optimal with 9 to 14 p.p.m. of zinc, and occurred with as little as 6 p.p.m., but not with 18 p.p.m. or more. Zinc could not be replaced by iron, copper, manganese, or molybdenum. No vesicles were formed if the pH was adjusted to 5.9 or 7.1, or if the gelatin was replaced by 2 per cent agar. A few were observed on 0.5 per cent agar at zinc concentrations of 11 and 23 p.p.m.

Obviously the operation of stimuli affecting fungal behavior is poorly

understood at present. The findings of Sharp & Smith do not invalidate those of Dickinson and others, because physical stimuli must have to evoke chemical responses in the cell before observable morphological and physiological changes follow. Such chemical responses might easily be brought about by other chemicals applied to the cell in place of the contact stimulus. On the other hand, the differences Dickinson found in response to waxes congealing at 52° and 36°C. may have been caused by their chemical composition rather than by their physical properties.

*Infection of susceptible hosts by specialized parasites.*—The early stages of infection by several fungi, including *Phytophthora infestans*, *Helminthosporium victoriae*, and *Colletotrichum linicolum*, resemble those of the rusts. The manner of penetration by these fungi has already been discussed (7, 9, 16), and it was pointed out that usually each produces an appressorium and infection peg that goes directly through the cuticle into the host epidermal cell. In susceptible varieties the infection enlarges into a so-called primary hypha that is globular in *P. infestans* and *C. linicolum*, with lobes or projections that soon develop into secondary hyphae. The primary hyphae of these two fungi occupy only the epidermal cell originally entered, while those of *H. victoriae*, which are elongate and thick, may penetrate into several epidermal cells. In this respect it resembles a similar structure produced by *Colletotrichum lindemuthianum* [Leach (11)]. Leach considered that this structure was not a vesicle, but rather the result of nutritional and spatial conditions on the fungus. Nevertheless, these structures seem to be characteristic and consistent in their appearance, and from them secondary hyphae arise that spread to adjoining cells, in much the same way that the infection hyphae originate from the substomatal vesicles in certain rusts. The secondary hyphae of these three fungi are principally intercellular, and no distinct haustoria are produced by *H. victoriae* or *C. linicolum*. *P. infestans* forms haustoria, but apparently only sporadically (66). In susceptible hosts the secondary hyphae of these fungi move more or less rapidly, and progress for some distance before obvious damage to the host ensues. Schwinghamer (7) found that *C. linicolum* required two days to penetrate and pass through the primary hyphal stage on the cotyledons of the susceptible flax variety, Punjab. At the end of four days the hyphae had reached the opposite epidermis and had expanded to form a circular lesion.

The primary hyphae of *H. victoriae* at first produced no noticeable effect on the invaded epidermal cells of the susceptible oat variety, Victoria, and secondary hyphae developed so rapidly that they often reached the opposite epidermis 24 hr. after inoculation, and completely occupied the intercellular spaces in 48 hr. (9). The reaction of the host cells was variable: some near the hyphae appeared unaffected while others at a distance were completely disintegrated. In the early stages many cells were apparently unaffected, but 48 hr. after inoculation the damage was extensive near the point of inoculation. As the hyphae spread, there were gradations between healthy cells in

the periphery of the lesion and dead cells near the center. *P. infestans* on the variety Cobbler also produced appreciable areas of dead tissue 48 to 72 hr. after inoculation (16). It is significant that all three of these pathogens caused necrosis of the host cells of susceptible plants more or less rapidly, but that the secondary hyphae were always in advance of the dead cells.

*Infection of resistant host by specialized parasites.*—The infection of resistant host plants by the rusts and powdery mildews, which are obligate parasites, and by such fungi as *C. linicolum*, *H. victoriae*, and *P. infestans*, is of interest academically as an approach to the understanding of the nutrition of obligate parasites. It is of interest practically because a knowledge of these processes may help to determine the manner of developing disease-resistant varieties. In the following discussions the term "resistant" is used to designate the reaction of a variety or host to a particular pathogenic race of the fungus in question, since in many cases the same variety may be susceptible to other races. Gaumann (1) applies the term "parabiotic" to host-pathogen combinations if the plant is resistant due to hypersensitivity to the race involved. Use here of the term resistant therefore implies a parabiotic combination.

The relation of cereal rusts to resistant host was studied by Stakman (13), Allen (67) and others whose work has been reviewed by Rice (61, 62) and Gaumann (1). Little in the way of histological studies has been published recently. The general picture of the reaction of resistant cereals to rust is that the host cell, being highly susceptible, or hypersensitive, to the fungus, dies soon after or during penetration by haustoria, resulting in the death of the fungus, by starvation or otherwise.

The reaction of barley to powdery mildew, *E. graminis* var. *hordei*, is described by White & Baker (30). The powdery mildews typically grow on the surface of the host plant, and send haustoria into the epidermal cells. As the first haustorium from a germinating spore starts to draw nutrients from the host (if it is susceptible) the superficial mycelium spreads and other haustoria are established. White & Baker (30) found that the conidia germinate normally on all varieties of barley and send a haustorium into an epidermal cell. On a highly resistant (immune) variety this is followed quickly by the collapse of the mesophyll cells beneath the invaded epidermal cells. Contrary to the evidence of previous workers, White & Baker found that, while some of the epidermal cells collapsed, these were fewer than the collapsed mesophyll cells, and that the mesophyll was affected first. In highly resistant hosts the collapse of the mesophyll cells halted the growth of the fungus so that usually no more than one haustorium was produced from a single germ tube. On resistant and semi-resistant varieties the effect on the mesophyll occurred later, so that about five and ten haustoria, respectively, were produced on these varieties before the death of the mesophyll halted the fungus.

When oats, rye, and certain Kenya varieties of wheat were inoculated

with *E. graminis hordei* (30), germ tubes, appressoria, and short infection papillae were formed, but there were no haustoria. Usually there was no evidence of host reaction, although occasionally an epidermal cell collapsed. Federation wheat reacted like resistant barley. It seems possible that this represents a type of resistance differing from that due to mesophyll reaction. It resembles the behavior of *H. victoriae* on certain oat varieties (9), and yet must be highly specific because the hosts not penetrated by *E. graminis hordei* are susceptible to other varieties of this species.

The reaction of resistant flax to *C. linicolum* resembles in some respects that of oats and rye to *E. graminis hordei*. Some races seldom form normal appressoria, and consequently seldom infect any variety. Those which do form normal appressoria penetrate susceptible varieties easily, but on resistant ones many appressorial pegs fail to penetrate. Whether this is a passive form of resistance due to the hardness of the cuticle [axeny of Gaumann (1)] was not studied. It is of interest that cellophane was easily penetrated, but this may be soft relative to cuticle. The fact that often the infection peg produced a hemispherical bulge with a brownish halo on the inside of the cell may indicate host reaction, but often this was not apparent. If the fungus penetrated, it was stopped sooner or later, depending upon when the host cells died. Sometimes the invaded epidermal cell died before the secondary hyphae could develop. Again, secondary hyphae might develop and spread to adjoining cells, but they seemed to be smaller and less numerous than in more susceptible varieties. Schwinghamer (7) notes that, on individual plants of moderately resistant and intermediate varieties, there is a range of reaction types from single necrotic epidermal cells to infections where the fungus spreads almost unimpeded.

The reactions of resistant oat varieties to *H. victoriae* apparently also may begin with spore germination (9). Race M 224 of *H. victoriae* on the variety Clinton produced only about 45 per cent of normal-appearing germ tubes. The rest produced abnormal appressoria or were irregular in some other respect. The walls of 25 per cent of the germ tubes dissolved, allowing the protoplasm to flow out on the leaf. A similar behavior was described by Dickinson (5) when urediospores of *P. tritici* and *P. graminis* were germinated on membranes made with wax congealing at 36°C. Those germ tubes of *H. victoriae*, which were otherwise normal, were short, the appressoria were sparse, and only about one-fourth of these effected penetration. About one-third penetrated through stomata and the rest at the junction of epidermal cells. Primary hyphae, whether in the epidermal cells or substomatal cavities, remained about the same size and shape as an appressorium and disintegrated soon after penetration, leaving the host cells apparently unharmed. This seems a clear case of a plasmatic defense reaction (1), and is very similar to the disintegration of certain mycorrhizal fungi in the cells of the tree symbiont.

On the Cornellian variety of oats, spores of the same race (M 224) of *H.*

*victoriae* germinated freely, but many of the germ tubes were irregular in shape and many collapsed. Appressoria were abundant but also abnormal. Unlike Clinton, the cells of Cornellian reacted sharply to the presence of the appressoria as a fibrous reorientation or increase in density of the protoplasm, which appeared as early as 18 hr. after inoculation. Later there was an amber discoloration of the walls directly below the appressoria. Penetration pegs grew between the walls of the epidermal cells, but penetration of cells was seen only once. About 72 hr. after inoculation, some of the older appressoria finally induced an amber color in the protoplasm, involving one to four cells adjoining the site of the appressorium. It was less apparent in later preparations, indicating that the cells apparently overcame the effects of the fungus and regained their normal condition.

Race DBC 12 of *H. victoriae* on the variety Cornellian germinated and grew rapidly although some hyphae disintegrated. Only about 5 per cent of the appressoria effected penetration and primary and secondary hyphae were produced. The host cells reacted by colorless changes in the cell walls, followed by an amber color 72 hr. after inoculation. Primary hyphae, which were found 18 hr. after inoculation, established secondary hyphae at 48 hr., and started to disintegrate at 117 hr., at which time the host cytoplasm was densely amber. After the disintegration of the primary hyphae, the host cytoplasm was only slightly colored. The secondary hyphae caused destruction of the protoplasts in cells at some distance, and those adjacent to the hyphae turned dark amber.

It appears from these studies that *C. linicolum* and *H. victoriae* invoke highly specific reactions in the hosts they attack. In this they resemble *P. infestans*, another nonobligate parasite that has been studied extensively by Müller (15, 68, 69). He found that potato tissue invaded by the fungus passes through five stages of degeneration leading to death. During the first three of these stages the host cells retain their turgor, and the fungus is still able to grow and sporulate. The last two stages are characterized by cell collapse and the infiltration of the cell walls and cytoplasm with phlobaphene-like compounds. This tissue will not support the growth of *P. infestans* or of any of several other fungi ordinarily able to grow saprophytically. Unless the fungus has grown beyond these dying and dead cells, its further progress is halted. In resistant varieties (i.e., parabiotic<sup>3</sup> combinations) the host cells pass through the first stages within one or two days, and the fungus being in the dead tissue, dies also. Such infections result in a minute area of dead cells, often invisible to the unaided eye. In susceptible varieties the affected cells may stay alive 6 to 14 days and the fungus is able to penetrate beyond the dead tissue, and so remain alive, and sporulate. Thus resistance and susceptibility depend upon the speed with which the host cells react, and this

<sup>3</sup> Gaumann uses the term "parabiotic" and Müller "parabiontic." They evidently mean the same thing.

in turn is determined by the genotype of the plant and that of the infecting fungus race. Müller found also (15) that *P. infestans* was able to penetrate a number of other species of plants which reacted like a resistant variety of potatoes after penetration.

The reaction of plants highly resistant to the rust, powdery mildews, and late blight has been described as hypersensitive or necrogenic (1, 69). From the facts available it appears that these terms may also be applied to the reaction of resistant hosts to *H. victoriae* and *C. linicolum*. As described by Müller (69) hypersensitivity is the rapid death of the host cells early in the process of infection, so early in some cases that infection in the sense of a "stable parasitic relation with the plant" is never achieved. Subsequently the parasite also dies, possibly of starvation in the case of the rusts or mildews, which are obligate parasites. Although *P. infestans* is a facultative saprophyte, the tissues it kills are unsuitable as a substrate. Müller says the tissues are fungistatic at first, and later fungicidal. If the host reaction is slow, the fungi may live longer, the degree of their development depending upon the reaction speed of the host. In the most susceptible varieties death of the host cells is much delayed, and a temporary compatible (eusymbiotic) relation between the host and pathogen is achieved. In an earlier paper Müller (70) suggests that invasion by certain parasites results in the death of adjoining cells, which in turn inhibits the growth of the pathogen. White & Baker (30) point out that this seems to be true of *E. graminis hordei* on resistant hosts, since the uninvaded mesophyll cells are the ones which react first. If the same phenomenon is responsible for the resistant reaction of oats to *H. victoriae*, it starts operation very early, because here the physiology of spore germination is definitely affected, in contrast with the case of late blight of potatoes, where resistant hosts are penetrated before any reaction occurs (15).

That tissue infected by certain fungi may become toxic to them is strongly suggested by the work of Yarwood (71). He found that infections by *Uromyces phaseoli*, involving part of a bean leaf, would render the rest of the leaf apparently immune to infection upon subsequent inoculation. The same was true of *Puccinia antirrhini* on snapdragon. Further experiments indicated that the effect was due to the production by the infected tissue of a toxic gas, which retarded the germination of the rust spores.

Fungi that live chiefly on dead tissue, after killing it in advance of the hyphae, can be said to be established as parasites as soon as they start to grow on such tissue. The infection process, therefore, is limited to penetration and sometimes to preliminary growth on tissue killed or weakened by some other agency. Recent studies of these phenomena have been noted.

*Effect of environment on infection by specialized parasites.*—This review does not include the effects of environment on plant disease, but does seem pertinent to point out in connection with the discussion of infection by specialized parasitic fungi that much attention has been paid lately to the ef-

fects of light, temperature and other factors on the reaction of cereals to rusts and mildews. This subject was very thoroughly reviewed by Hart (18) in 1949. Recent papers (72, 73) emphasize further the fact that certain race-variety combinations involving the rusts and powdery mildews often result in different reaction "types" at different temperatures and light intensities: in some, high temperatures result in increased susceptibility, while others are not affected. This complicates the task of breeding for resistance to these pathogens. It should also provide a means of manipulation in studies of the nutrition of obligate parasites.

#### INFECTION AND DISEASE CONTROL

Knowledge of the mechanism of infection by plant pathogens is important in control of the diseases that they produce. Avoidance of wounding or injuring plants is an old principle; yet occasionally new practices lead to the opening of unsuspected avenues of entrance (32, 34, 35, 36, 48, 49) and previously unknown relationships involving mechanisms of infection are found (47, 48, 49, 50). Wagener & Davidson (31) point out that "much more is known concerning the chief places of entry of heart rot fungi into trees than the details of inception of the resulting decay." Problems of this type, which involve the early stages of infection, are much in need of investigation.

Knowledge of the phenomena of infection is perhaps of even greater importance in the development and selection of disease-resistant varieties. Highly specific reactions of the hypersensitive type, resulting in practical immunity, was welcomed by plant breeders and plant pathologists alike (74). Although its ultimate cause is obscure, such resistance was easy to determine in breeding material, was often simple in inheritance, and was thought for some time to be relatively unaffected by environmental fluctuations. But the results have sometimes been disappointing because of the frequency with which new races arose in some of the most destructive fungi. Where a high degree of sensitivity to a pathogen existed, the opposite of hypersensitivity [eusymbiosis of Gaumann (1)] also was not only possible, but highly probable and, of course, resulted in susceptibility when it occurred.

Efforts to utilize less specific types of resistance have been made, especially by workers trying to get late blight-resistant potatoes, because hypersensitive varieties are attacked by eusymbiotic races very quickly after they come into existence. Potato clones which are susceptible (non-hypersensitive) to all known races but which survive epidemic conditions longer than others have been known for a long time; their disease reaction is referred to as "partial resistance," "minor gene resistance," "field resistance," etc. Neiderhauser & Mills (75) emphasize the usefulness of varieties with such resistance in Mexico, where the climate is very favorable for blight, and where all known parasitic races apparently exist. Müller & Haig (76) emphasize that the "field resistance" of such varieties limits the speed with which epidemics develop. This would limit the importance of blight in years when



the weather was only moderately favorable, and make possible easier control with fungicides. The value of such resistance is shown also by the severity of stem rust of wheat in the spring wheat area of the United States in the epidemic years of 1953 and 1954. Although both durum and bread wheats are equally susceptible (in terms of reaction type) to the prevailing race (15 B) of *Puccinia graminis tritici*, the durum was damaged much more than the common wheats.

Other attempts to make use of the non-hypersensitive types of resistance are being made. Rosen (77) found that certain oat varieties had fewer pustules than others. Smith & Blair (78) refer to "adult plant" resistance to mildew in wheat, and show that it is inherited and therefore can be used in breeding.

Resistance of this type is generally complex, and not always due to differences in ease of penetration or infection, although the great importance of these factors was shown by Hart (79, 80) some time ago, and other instances are mentioned in her review (18). Müller & Haig (76) showed that "field resistance" of potatoes to late blight was attributable, in part at least, to fewer infections on the upper surfaces of the leaves of resistant plants. There was no difference in the ease of infection of the lower surface, and the reason for the resistance of the upper surface was not determined.

Recent contributions mentioned in this review indicate that penetration and infection of plants might be limited by resistance to bruising (36), age of the leaves (20, 21), and chemical barriers (19, 20). The importance of water congestion to infection as shown by Johnson (25, 26, 27) and the demonstrated differences in the tendency of varieties to become water-congested (81) suggests that this is also a factor in disease resistance. This is illustrated by Johnson's finding that, if potato varieties were kept in the moist chamber for 24 hr., they were equally susceptible to late blight (26). His study included the Sebago variety, which, though susceptible to infection by all known parasitic races of *P. infestans*, has a certain degree of "field resistance." Apparently the long period in the moist chamber broke down this resistance. However, if the plants were kept in the chamber for 6, 9, or 12 hr., the number of infections was fairly proportional to the resistance or susceptibility of the varieties. Johnson (27) points out that other factors, such as stomatal behavior, may play a role in these results; however, the association of increased penetration with water congestion also occurs with pathogens that penetrate principally through the cuticle (*C. lindemuthianum* and *P. infestans*) rather than through the stomata, as rusts do.

It is generally assumed that resistance caused by mechanical or other barriers to infection would be effective against a broader range of races than is hypersensitive resistance. Although data are insufficient, this is to some extent supported by experience. For example, when a variety of potatoes is inoculated with a mixture of races of *P. infestans* to which it is equally susceptible (non-hypersensitive), after a number of generations one race pre-

dominates over the others (82). The same has been shown to be true of races of *P. graminis tritici* on wheat (83). The cause of such differential survival is not known. Castronovo (84) has shown that isolates of *P. infestans* differ in the number of infections which they can cause from identical inoculations. In spite of the many factors involved, and the lack of knowledge about them, the tendency to use less specific types of resistance seems reasonable and useful.

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## NEISSERIA AND NEISSERIAL INFECTIONS<sup>1</sup>

BY HENRY W. SCHERP

*Department of Bacteriology, University of Rochester School of  
Medicine and Dentistry, Rochester, New York*

Since the advent of successful chemotherapy for meningococcal disease and gonorrhea, the genus *Neisseria* has tended to become a Cinderella in bacteriological research. Such vogues in research seem especially unfortunate in this case for the group still offers an abundance of challenging fundamental problems in nutrition, metabolism, immunochemistry, and parasitism. Hill (1) has analyzed in detail unsolved problems of the biology of the gonococcus and of the host response to it. Similar considerations apply to the other *Neisseria*. Moreover, neisserial diseases, have not become negligible. During the latest epidemic years of 1943-4 in the United States, meningococci caused nearly 6,000 deaths, or from two to seven times as many as such diseases of active public health interest as measles, acute rheumatic fever, poliomyelitis, diphtheria, typhoid and paratyphoid fever, and scarlet fever [Hedrich (2)]. In the interim, an annual total of about 1,000 deaths keeps meningococcal disease even with the others. Meanwhile, the incidence of reported cases of gonorrhea in the United States has exceeded that of the pre-sulfonamide era. The rate rose from 128 per 100,000 in 1935 to a peak of 284 in 1947 and has recently stabilized around 160. The annual total, therefore, is about 250,000 (3, 4).

In the circumstances, an "annual review" would be a meager offering. The present account, therefore, aims to cover not only recent reports but also earlier work either not appropriate for the standard texts or treated only briefly in them. An excellent general account of the meningococcus and gonococcus has been presented by Smith & Conant (5). Other recent useful accounts, particularly in relation to pathogenicity, have been given by Schoenbach, Mahoney & Thayer (6) and Burrows (7). A detailed discussion of earlier work is given by Wilson & Miles (8).

### TAXONOMY

*Serological relationships of the meningococcus.*—Probably the most widely accepted classification of meningococci has been based upon the four serotypes of Gordon & Murray (1915). Their Types I and II have accounted for a large majority of strains and correspond closely to the Types A and B of the French system, introduced by Nicolle, Debains & Jouan in 1918. Eventually, it became evident that a practical distinction could not be maintained between Types III and I. Type IV occurred rarely and seemed indistinguishable from the French Type B. Types C and D had no correlate

<sup>1</sup> Except for a few journals that were not received in time, this review covers the literature through December, 1954.

in the British system. Because of their overlapping agglutination, Type II strains were frequently designated as "Group II." Then in the American epidemic of 1935-7, encapsulated strains were encountered commonly, which agglutinated to some degree with Group II antisera and were therefore designated as Group II *alpha*, but which were found later to form a distinct and relatively homogeneous serological group. Branham (9) has recently reviewed this subject and related epidemiological matters in detail and has presented a revised classification, recommended by an international committee, which accommodates the interim developments and provides for future developments. The correlation of the new Groups A, B, C, and D with earlier classifications is shown in Table I.

TABLE I  
RELATIONSHIPS AMONG THE VARIOUS CLASSIFICATIONS OF MENINGOCOCCI\*

Dopter and Pauron, 1914	Rockefeller Institute (Wollstein, 1914)	Gordon and Murray, 1915	Griffith and Scott, 1916	Pullon, 1917	Nicolle, Debains, and Jouan, 1918	Evans, 1920 (Tropins)	Common Use Since 1940	Recommended by Committee 1950
Meningococcus	Normal	I	I	C	A	R	I	A
	Irregular	III		A				
Parameningococcus	Parameningococcus	II	II	B	B	S	II	B
$\alpha, \beta, \gamma$		IV	II		B	Z	IV	D
					C		II <i>alpha</i>	C
					D†			

\* From Branham (9).

† Relationship of this D to other groups is unknown.

Of great general interest is the transformation of meningococcal serotypes by Alexander & Redman (10). Rough (R) variants derived from Type II *alpha* smooth (S) strains were converted to Type II *alpha* S and to Type I S when cultured in the presence of partially purified deoxyribonucleic acid (DNA) from the respective S types. Of special interest was the direct conversion of a Type II *alpha* S to a Type I S. Such transformations have typically had to be accomplished via R variants. The transforming capacity of the DNA preparations was destroyed by crystalline deoxyribonuclease. However, when this enzyme was added to the test culture as soon as 15 min. after the addition of DNA, but not sooner, the transformation was not impeded, *i.e.*, the essential reaction between the transformable cells and the DNA occurred rapidly and irreversibly even before cell division began.

Evans (11) reported that on prolonged cultivation on human blood agar, a Gordon Type III meningococcus gave a stable R variant that had lost the power to ferment maltose. Biochemically, such a variant is a gonococcus!

Keogh, North & Warburton (12) found that meningococcal polysaccharide rendered erythrocytes agglutinable by the homologous antibody. Podliachouk & Eyquem (13) have observed that certain equine antisera to Groups A and C meningococci agglutinated certain equine erythrocytes, especially of equine blood group A. Absorption tests showed that the responsible antigen was neither equine blood group antigen A nor F.

*Serological relationships of the gonococcus.*—Despite much painstaking effort, serological investigation of the gonococcus has not been very rewarding. Nevertheless, diagnostic tests for complement fixation by the sera of patients and gonococcal antigens ("gono-reaction") appear to be in continued use in Europe [Reyn (14); Le Minor (15)]. Saint-Prix (16) has described a flocculation test based on a gluco-lipid extract of gonococci. The accumulated evidence indicates that freshly isolated or smooth phase gonococci may be divided into two major serotypes, though an immunological *tour de force* may be required. On culture, gonococci may change quite readily to variants that have lost surface antigens and can be definitely distinguished from the parent strains (8). Three modern investigations seem to merit discussion here. Uroma (17) tested the type specificity of gonococcal polysaccharides by complement fixation with rabbit antisera. Formamide extraction of washed gonococci gave only serologically inactive polysaccharide, so the author returned to the taurocholate extraction used earlier by Casper (18). Protein and taurocholate were precipitated by acidification and the polysaccharide was precipitated from the supernate by ethanol. Purification was effected by removal of water insoluble matter and reprecipitation by ethanol alternately from acid and alkaline solution. The product was strongly anticomplementary unless reprecipitated repeatedly. Starting with antiserum to a single strain, then preparing antiserum against a strain whose polysaccharide did not react with it, and so on, he sorted 30 strains into 4 groups by using 4 antisera: A, 18 strains; B, 7 strains; C, 3 strains; D, 2 strains. Though based on so few antisera, these results confirm earlier findings of two major serotypes. Reyn (14) investigated the complement fixation reaction with heat-killed cells of 700 strains of gonococci. Since the sera from 70 per cent of normal adult rabbits gave positive reactions, this animal was unsuitable for antisera. A rising incidence with age, the isolation of *Pasteurellae* from the noses of rabbits, cross reactions, and cross absorptions indicated that these reactions were attributable to antibodies formed against the *Pasteurellae* carried by the majority of rabbits. Guinea pig sera were free from this difficulty and were used in the later work. The common antigen was shown to be the species antigen shared by all gonococci. Although sera absorbed with gonococci were frequently anticomplementary, it was possible to show that gonococci contained also a number of type- and strain-specific antigens. Simple cross absorptions, however, indicated an extreme degree of hetero-



geneity. It was not always possible, for example, to establish the identity of two strains isolated from the same patient at different times. An effort was made to develop "factor" sera, similar to those used for *Salmonella* typing, by absorption with carefully selected strains of gonococci. With one group of 5 factor sera, it was possible to sort more than 100 strains chosen at random into 27 types. However, cross absorptions showed that frequently strains of the same type still differed in minor antigens. Sometimes even strains isolated simultaneously from different sites (e.g., cervix and urethra) of the same patient differed when tested by the factor sera. Very recently, Chanarin (19) has used an indirect hemagglutination technique. All gonococci were used within a week of their isolation. To increase the specificity of antisera, only two injections of vaccine were given, three days apart, and the rabbits were bled seven days later. For the tests, washed gonococci were dissolved in dilute alkali, the solution was neutralized and treated with five volumes of ethanol, and the water soluble portion of the resulting precipitate was used as antigen. These preparations were very toxic and heat stable and gave rise to gonococcal agglutinins when injected into rabbits. The active principle may have been the Boivin-type antigen [Boor & Miller (20)]. The antigen was readily adsorbed by erythrocytes, which were then tested for agglutinability by antigenococcal sera. Cross absorption tests with 18 strains and their antisera revealed two serotypes. Fifteen strains absorbed all antibody from all sera; the other three strains absorbed each other's antisera completely but removed only a part of the antibody from the other 15 antisera. The first group was assigned the antigenic formula A, B; the second group, A. In all, 59 of 67 randomly selected strains were Type I. Ancillary experiments indicated that the antigens concerned were distinct from those involved in the usual agglutination and complement fixation tests with whole gonococci. Similar extracts of eight strains of meningococci gave complete cross absorption with Type I gonococcal antiserum. With one exception, extracts of nonpathogenic *Neisseria* did not react with this serum. In conformity with the earlier observations of others, prolonged cultivation yielded R variants, which no longer gave mucoid growth and had lost antigens A and B. Extracts of the R forms, however, reacted in the hemagglutination test with R antisera. In view of the cross reactions with meningococci, it is evident that even this promising investigation has not revealed specific gonococcal antigens. Considering the relative difficulty of inducing gonorrhea experimentally in humans with cultured gonococci, as compared with gonorrheal exudates, it is doubtful that any cultured gonococci studied to date have contained the full antigenic armamentarium employed by this organism *in vivo* [Mahoney, Van Slyke, Cutler & Blum (21); Hill, Huffer & Nell (22)]. Of interest in this respect is the finding by Morton (23) of small colony forms of gonococci in three out of 135 cases of gonorrhea. These grew poorly in fermentation media and, possibly on that account, did not ferment glucose. On repeated subculture, they dissociated into conventional gono-

cocci. Reyn (24) found what seem to be the same forms in one per cent of isolates from gonorrhea in Denmark.

*Other Neisseria.*—Pelczar, Hajek & Faber (25) described a homogeneous group of gram negative diplococci of neisserial morphology, isolated from the pharyngeal regions of guinea pigs and provisionally named *Neisseria caviae nov. sp.* All gave the oxidase reaction. Some were weakly hemolytic. They did not ferment glucose, maltose, sucrose, fructose, or mannitol. They differed, however, from *N. catarrhalis* and from other chromogenic *Neisseria* because of the distinctive light-caramel to dirty-brown color of their colonies. Berger (26) described the isolation of hemolytic strains of *N. perflava* from human saliva. Warner, Faber & Pelczar (27) have made a serological study of some of the nonpathogenic *Neisseria* species identified by the conventional criteria of fermentation and chromogenesis. The nonchromogens, *N. catarrhalis* and *N. sicca*, were serologically distinct. The chromogens formed three separate groups: fermentative (*N. flava* and *N. perflava*); nonfermentative (*N. flavescens*); and nonfermentative (*N. caviae nov. sp.*). Since *N. flava* and *N. perflava* could not be sharply distinguished serologically, the authors suggested that they be lumped and renamed *N. pharyngis* despite fermentative differences. Morris (28) has recently reported on 37 biochemical and cultural characteristics of each of 87 strains of *Neisseria* isolated from seven carious and four noncarious human mouths. The conventional nonpathogenic species were recognized but the group was heterogeneous and was sorted into 30 types. The predominant types from the noncarious mouths were chromogenic, produced polysaccharide copiously on 5 per cent sucrose (see discussion below on metabolism of *N. perflava*), and frequently did not produce catalase. The opposite was true in carious mouths. Peculiarly, many of the types gave a negative oxidase test. Though knowledge of the various reactions is desirable, one doubts the value of splitting the group into so many types on the basis of so many characteristics.

*Anaerobic gram negative cocci.*—The anaerobic gram negative cocci have not been studied intensively, although the literature contains numerous sporadic reports of their association with various pathological processes. Douglas (29) found *Veillonella gazogenes* (*Micrococcus lactilyticus*) to be one of the most numerous organisms in saliva. The count ranged from 3 to 340 million per ml. in the salivas of 17 persons, or nearly as great as that of streptococci and facultatively anaerobic micrococci combined. Breed, Murray & Hitchens (30) recognized two genera: anaerobic *Neisseria* (diplococci) and *Veillonella* (clusters). The validity of *V. gazogenes*, the most well-known species, was challenged by Foubert & Douglas (31) because their strains were gram positive during the first part of the growth cycle. They proposed reclassification as *Micrococcus lactilyticus*. Johns (32), working with isolates from the rumen of sheep, confirmed their observation but Langford, Faber & Pelczar (33) and Morris (28) did not. This sort of situation emphasizes the shaky basis of much bacterial taxonomy. The gram variability of the *Neis-*

*seria*, for example, is notorious and the matter is not much more satisfactory with some of the gram positive cocci [(8) pp. 532, 562, 609]. Mitchell & Moyle (34) found that a strain of *N. catarrhalis* was quite gram positive when young and contained relatively large amounts of an unidentified phosphate ester, which they believed to be correlated with the staining and with penicillin sensitivity. If comparisons are to be valid, cultures should be grown in standard media under standard conditions, they should be stained at exactly defined stages of the growth cycle, and a standard technique of the Gram stain should be used. Langford *et al.* (33) seem to have made a good start on the *Veillonella* problem with a study of 49 strains of anaerobic gram negative cocci isolated at random from the salivas of 51 healthy persons. All grew as diplococci of typical neisserial arrangement, not in clusters. All were gram negative in 8-hr. cultures, as were two strains of Foubert's *M. lactilyticus*. The group was remarkably homogeneous: they formed gas in peptone; did not ferment glucose, galactose, fructose, sucrose, lactose, maltose, manitol, or inulin; fermented lactate; did not liquefy gelatin; decolorized litmus milk; produced hydrogen sulfide but not indole; reduced nitrate to nitrite; were nonhemolytic and oxidase negative. Three strains produced catalase. The same results were obtained with strains of *V. gazogenes*, *V. parvula*, *V. alcalescens*, six unidentified species of *Veillonella*, and *M. lactilyticus* received from other workers. The anaerobic *N. vulvo-vaginitis* of Reynes (35) was found to grow in chains. The authors suggested that the description of the genus *Veillonella* be amended to read "anaerobic gram-negative diplococci" and that the generic designation of "anaerobic *Neisseria*" for such organisms be abandoned. Recently, Morris (28) has determined 17 biochemical and cultural characteristics of each of 59 strains of *Veillonella* from 11 human mouths. On this basis, the strains could be divided into 14 types. *V. gazogenes* and *V. parvula* comprised half the strains.

#### PHYSIOLOGY

*Cultural requirements.*—There is a steady production of new brews for the more efficient primary isolation of the *Neisseria*, especially for the gonococcus. Since most of the reports are not comparative, it seems that an experienced bacteriologist can obtain equally satisfactory results with a variety of media. Carpenter and 12 others (35) made a simultaneous comparative test of 209 specimens on 12 media. In all, 70 strains of gonococcus were isolated. The three best media, which yielded 65, 63, and 63 strains respectively, were (a) a modified McLeod's agar with Nile Blue A and enriched with horse plasma and hemoglobin, (b) GC agar base, experimental, with hemoglobin and supplement B (a thermolabile yeast extract) (all Bacto), and (c) Proteose No. 3 agar (Bacto) with Nile Blue A and enriched with horse plasma and hemoglobin. Lankford (36) replaced yeast extract successfully in proteose-peptone-hemoglobin agar by adding one volume per cent of a solution containing 20 per cent glucose, 0.25 per cent glutamine, and 0.0001 per cent cocarboxylase, sterilized separately [by filtration]. The problem of preserving

gonococci during transport to the laboratory has been reviewed recently by Stuart, Toshach & Patsula (37). They emphasize three principles: the use of swabs treated with charcoal to neutralize an inhibitor present in agar, prevention of oxidation and desiccation of material on swabs during transport, and absence of nutrients in the transport vehicle to prevent multiplication of adventitious organisms.

The specific nutritional requirements of gonococci and meningococci are less complex than they were expected to be. Boor (38) found that meningococci grew abundantly on a medium based on agar, glucose, and a tryptic digest of casein but that from 250 to 750 mg. of cystine per liter had to be added to support the gonococcus. Lankford (36), in summarizing previous work from his laboratory (39, 40), found that 23 per cent of 1042 freshly isolated gonococci required the addition of glutamine to a proteose-peptone hemoglobin agar, 0.8 per cent required co-carboxylase (they could not phosphorylate thiamine but could use thiamine monophosphate), and 0.5 per cent needed unidentified growth factors. Glucose and cystine increased the size of colonies but cystine was not needed if the glucose was not autoclaved. Gould (41) found that gonococci acquired a dependence on glutathione when subcultured repeatedly in its presence, as on the conventional chocolate agar. Welton (42) showed that this phenomenon accounted for the loss of ability to grow in a chemically defined medium [Welton & Scherp (43)]. Gould, Kane & Mueller (44) cultivated several strains of gonococci on a medium containing only agar, glutamic acid, histidine, glutathione, glucose, starch, and salts. Casein hydrolyzate or meat infusion stimulated growth but were not essential. Welton, Stokinger & Carpenter (45) grew 45 of 60 strains in a liquid medium containing eight amino acids, indole-3-acetic acid, glucose, and salts, incubated with supplemental carbon dioxide. Landy & Gerstung (46), Hill (1), and Lankford [quoted by Hill (1)], however, were not successful with this medium. Sensitivity to inhibitors, rather than complexity of nutritional requirements, may account for much of the fastidiousness of the gonococcus. Subsequently, Welton (42) experienced difficulty in starting growth from small inocula, possibly owing to inhibition by heavy metals or fatty acids, for her medium was satisfactory after adsorption with calcium phosphate (43). Ley & Mueller (47) showed that starch was required in solid media (44) to adsorb an inhibitor in agar, probably a free fatty acid, which could be removed only by prolonged extraction with hot methanol. Lwoff (48) believed that serum or cholesterol, needed for the growth of certain strains of gonococci in a broth, acted by complexing with some inhibitor.

Frantz (49) grew several strains of meningococci in a liquid medium containing only glutamic acid, cystine, glucose, sodium, potassium, and ammonium chlorides, sodium phosphate, magnesium sulfate, and supplemental carbon dioxide. Grossowicz (50) obtained similar results: pyruvate and lactate could replace glucose, calcium was necessary, but cystine was inhibitory. Strain differences and selective phenomena complicate the picture. Scherp &

Fitting (51) found that some freshly isolated strains grew well from minute inocula on the first subculture in Frantz medium, whereas some "laboratory" strains required heavy inocula. After but one passage in Frantz medium, however, all strains tested grew from minute inocula even in the absence of glucose. None of the simple media was optimal, for on substitution of a "vitamin-free" casein hydrolyzate for glutamic acid, even a recalcitrant strain grew from small inocula on the first passage and others produced from two to six times as much growth.

The nutritional requirements of other *Neisseria* are not as simple as expected. Ordal & Busch (52) found that 14 strains of *N. sicca* either required or were greatly benefited by biotin. Fitting & Scherp (53) could not grow a strain of *N. catarrhalis* in Frantz medium (49) but succeeded when a mixture of amino acids equivalent to casein was added. Nemes, Pelczar & Doetsch (54) found that 47 of 57 strains of nonpathogenic *Neisseria* required biotin or niacin or both for growth in a vitamin-free casein-hydrolyzate glucose medium. Three strains of *N. caviae* nov. sp. required also thiamine, pyridoxine, and pantothenate. In general, growth was still better in media made from natural products, such as trypticase soy broth. Martin, Pelczar & Hansen (55) showed that putrescine was needed by *N. perflava* in a medium containing 18 amino acids, biotin, and salts. Putrescine must be present in hydrolyzed casein. Rosebury, Gale & Taylor have made the very interesting observation that *N. catarrhalis* is stimulated when grown on blood agar as a satellite of *Streptococcus mitis*, *Str. faecalis*, *Micrococcus albus*, *Escherichia coli*, or *Candida albicans* (56).

It has long been recognized that most gonococci and meningococci require, or benefit by, a concentration of carbon dioxide greater than atmospheric. Concentrations from 2 to 22 per cent are equally effective, hydrogen, nitrogen, and carbon monoxide are inefficacious, and part of the benefit derives from the increased humidity resulting from incubation in closed containers [Ferguson (57)]. Tuttle & Scherp (58) found that meningococci varied widely in their need for supplemental carbon dioxide when grown in Frantz medium (49). The requirement of the most dependent strain could be met by adding 100 mg. of yeast autolyzate per liter or by much larger amounts of a mixture of amino acids, purines, and pyrimidines, and B vitamins but not by "V" factor, thioctic acid, or several intermediates of the tricarboxylic acid cycle. No supplement was a complete substitute for carbon dioxide, for the organisms died rapidly under vigorous aeration. Using a more sensitive version of the same test system, Larson & Scherp (59) found that yeast autolyzate could be replaced by an equivalent amount of amino acids. Most probably, the substitutes stimulate carbon dioxide production and also can substitute for it in certain reactions. In some vital reaction, however, carbon dioxide is irreplaceable.

*Action of drugs.*—Love & Finland (60) have measured the susceptibility of 50 recently isolated strains of meningococci to 11 antibiotics and sulfadiazine *in vitro*. Weight for weight, penicillin was by far the most active and

was as potent as in tests in the same laboratory in 1945 and earlier. The strains varied only within a narrow range in their sensitivity to each antibiotic but the sensitivity to sulfadiazine varied as much as a thousandfold. Six strains were moderately resistant to sulfadiazine but the remainder (88 per cent) succumbed to concentrations that can be maintained in the blood and spinal fluid. Since this was an interepidemic period, there was a preponderance of Group II strains, which have been consistently more resistant to sulfadiazine. The most recent similar data on gonococci seem to be those of Gocke, Wilcox & Finland (61), who found penicillin to be the most potent and sulfadiazine the least. Miller & Bohnhoff (62) showed that meningococci acquired resistance to penicillin by repeated passage on solid media containing increasing concentrations of the drug, then lost it on repeated subculture in penicillin-free media. Morphological abnormalities of resistant strains (greatly enlarged, deeply staining cells grouped in tetrads; colonies yellowish, smoother, stickier, stringier, and less translucent than normal) persisted through 10 subcultures in the absence of penicillin, after which the appearance returned to normal. Resistant strains did not produce penicillinase, were antigenically unchanged, but less virulent for mice, in which they produced an infection with increased resistance to penicillin. The same authors (63) described the development by spontaneous mutation of two types of streptomycin-resistant meningococci, one giving large yellowish colonies and still virulent for mice, in which they produced a streptomycin-resistant infection, the other giving small pearl-gray to yellowish colonies that grew only on media containing streptomycin and were virulent only for mice treated with the drug. The physiology of the latter type should be very revealing. Goeters (64) reported recently that certain proportions of penicillin and streptomycin act synergistically against meningococci *in vitro* and in experimental murine infection. Mills (65) found that the logarithm of the concentration of penicillin resisted by gonococci increased linearly with successive passages in broth containing graded doses of the drug. He interpreted the result to indicate the expansion of a single enzyme to overcome inhibition by the drug, rather than replacement of a partially blocked enzyme by another. Landy & Gerstung (46) found that sulfonamide-resistant gonococci generally produced more *p*-aminobenzoic acid in proportion to their resistance but the correlation was not good enough, nor was the amount produced large enough, to account definitively for the resistance. Hudemann (66) reported that sulfonamide-resistant strains of gonococci from patients did not change their resistance during 20 passages on ascitic-fluid blood agar containing from 1 to 10 mg. of *p*-aminobenzoic acid per 100 ml. Hagerman (67) found that at 37°C. a rise of 2.5°C. increased the sensitivity of gonococci to penicillin from two- to fourfold. Koch (68) made the very interesting observation that *beta*-progesterone is lethal to gonococci and meningococci in a concentration of ca. 1:25,000 at pH 7.4 and to gonococci at 1:1,000,000 at pH 6.8.

*Metabolism.*—Tonhazy & Pelczar (69) have reviewed and greatly ex-



tended previous investigations of the oxidative and associated mechanisms of the gonococcus. Among substrates of the tricarboxylic acid cycle, only those from  $\alpha$ -ketoglutarate through pyruvate were oxidized. An oxalacetate decarboxylase was demonstrated. Small amounts of citrate may be formed. Of the known amino acids, only D- and L-glutamic were oxidized rapidly. Histidine was deaminated. There was no appreciable oxidation, decarboxylation, deamination, or keto-acid formation from the others. A glutamate-aspartate transaminase was present but not glutamate-alanine. Penicillin had no effect on any of the numerous oxidations on which it was tried. The authors concluded that glutamate was oxidized mainly through the usual intermediates of the tricarboxylic acid cycle, ending with acetate, which the gonococcus cannot oxidize.

Randles (70) studied the oxidation of fatty acids by an unusual strain of *N. catarrhalis*, which could grow with acetate and lactate as the sole sources of carbon in a synthetic medium and did not oxidize glutamate or glucose. The similarity in rates and extent of oxidation of the even-numbered, straight-chain saturated fatty acids indicated a common mechanism. Propionate was oxidized slowly, formate not at all. The latter portions of the rate curves for the  $C_6$  and  $C_7$  acids resembled that for propionate, indicating that the latter accumulated as an intermediate. The results were consistent with the theory of  $\beta$  oxidation.

Pelczar & Doetsch (71) discovered several pharyngeal *Neisseria* that fermented maltose but not glucose. Glucose was not utilized, as shown by the determination of reducing substances and the failure of washed cells to consume oxygen in its presence. In contrast, *N. sicca* was shown by both methods to be very active on both sugars. Fitting & Scherp (72) found that nine strains of meningococci produced from 1.4 to 8.8 times as much acid from maltose as from glucose when grown in Frantz medium (49). The strain showing the greatest differential grew faster and effected the disappearance of inorganic phosphate with maltose and consumed it twice as fast as glucose. Similar results were obtained with washed cells (73). The rate of respiration with maltose was many times that with glucose. Respiration was stimulated by the presence of 3 per cent carbon dioxide in the gas phase. Anaerobic glycolysis was relatively very slow. Repeated subculture in the chemically defined medium yielded variants with increased activity for glucose. Cell-free extracts phosphorylated maltose, apparently to glucose and a glucose-1-phosphate (74), but effected no disappearance of phosphate with glucose or a number of other carbohydrates. Fitting & Doudoroff (75) showed that this phosphorylation of maltose was accompanied by a Walden inversion to yield  $\beta$ -D-glucose-1-phosphate and glucose and that the reaction was reversible. The equilibrium favored the synthesis of maltose. D-xylose could be substituted for glucose in the reverse reaction to yield  $\alpha$ -D-glucosido-xylose. Fitting & Putman (76) used this reaction to synthesize maltose labeled with  $C^{14}$  in the reducing or nonreducing glucose component by starting with  $C^{14}$ -labeled glucose or  $C^{14}$ -labeled  $\beta$ -D-glucose-1-phosphate, respectively.

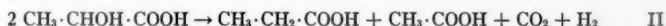


Hehre & Hamilton (77) described the first biosynthesis of glycogen-starch polysaccharides without the mediation of glucose-1-phosphate. Pharyngeal cultures of 12 healthy persons all contained strains of *N. perflava* that carried out the over-all reaction:



Washed cells and culture supernates also carried out this reaction, the former giving an amylopectin-like product, the latter giving a glycogen-like product. The mechanism is apparently a transglycosidation of the glucosidic radical from sucrose to a nidus of polysaccharide. Some iodophilic polysaccharide was formed also from glucose-1-phosphate. By selective inactivation, Hehre (78) showed the presence of both an amylsucrase and a phosphorylase, acting on sucrose and glucose-1-phosphate, respectively. A polysaccharide prepared similarly by Barker, Bourne & Stacey (79) had properties intermediate between amylopectin and glycogen, with an average chain length of 11 or 12 glucose radicals.

*V. gazogenes* is one of the few bacteria that ferment lactate, according to the over-all reaction (31):



Johns (32) found that washed cells attacked pyruvate, oxalacetate, L-malate, and fumarate, and decarboxylated succinate quantitatively to propionate and  $\text{CO}_2$ . During fermentation of lactate,  $\text{C}^{13}\text{O}_2$  was fixed in the carboxyl group of propionate and the yield of propionate was influenced by the concentration of  $\text{CO}_2$  present. He concluded that propionate was formed by the sequence: lactate  $\rightarrow$  pyruvate; pyruvate +  $\text{CO}_2 \rightarrow$  oxalacetate  $\rightarrow$  malate  $\rightarrow$  fumarate  $\rightarrow$  succinate  $\rightarrow$  propionate +  $\text{CO}_2$ .

#### PATHOGENESIS

At the risk of oversimplification, one concludes that the pathogenicity of gonococci and meningococci is determined by antiphagocytic and endotoxic mechanisms (5 to 8). Although quite readily ingested by phagocytes, they resist intracellular digestion and destroy the phagocytes if they are sufficiently virulent or numerous. Both contain a toxic glucolipid antigen of broad serological reactivity, not found in *N. catarrhalis* and *N. sicca* [Boor & Miller (80)]. The carbohydrate moiety of this antigen appears to be more specific. The data suggest that the glucolipid accounts for only part of the toxicity of the "nucleoprotein complex" of the cells. Acquired immunity to meningococci depends mostly upon antibodies to type-specific capsular or surface antigens, which prepare the cocci for lysis by complement and for disposal within phagocytes. Antibody to the glucolipid, however, confers a definite resistance to experimental murine infection (80). The type specific agglutinin response is short-lived [Dowling *et al.* (81); Falk & Appelbaum (82)]. Unpublished experiments by the present writer indicate that the blood of only one out of four adults is significantly bactericidal for Type I meningococci and that this activity can be neutralized by addition of homologous polysaccha-

ride. Acquired immunity to gonococci is tenuous. Mahoney *et al.* (21) concluded that an attack of gonorrhea conferred a significant resistance to experimental reinfection in human male volunteers. Nørsgaard (83) found that the gonococidal power of human serum was normally weak, increased after from one to two weeks of gonococcal infection, and remained elevated for as long as nine months after recovery, but did not correlate with the complement fixation test.

*Action of endotoxin.*—Although neisserial endotoxins have not been as well-defined as those of many other gram negative bacteria, the toxic effects of meningococci and gonococci resemble those produced by the class of endotoxins whose nature and physiological effects have been reviewed by Burrows (84) and Thomas (85). The thrombosis, vascular damage, and hemorrhagic necrosis that characterize fulminating human meningococcemia (86) strongly suggest the Schwartzman reaction. Black-Schaffer, Hiebert & Kerby (87) showed that living or dead washed meningococci were more potent than their culture filtrates in effecting the local Schwartzman reaction in rabbits. Strains from human purpuric meningococcemia seemed to contain an increased amount of the preparatory factor. In a number of rabbits, from five to eight intravenous injections of living meningococci at intervals of three hours produced purpura, which was regarded as a cutaneous manifestation of a generalized Schwartzman reaction, the hallmark of which, bilateral cortical necrosis of the kidney, was found also. The latter lesion, however, has not been described in human meningococcal disease. Adrenal lesions in the rabbits were considered to be a by-product of a general toxemia and not a Schwartzman reaction. Lithander (88) produced a syndrome of acute adrenal insufficiency in rabbits by meningococcal, diphtherial, staphylococcal, and streptococcal toxins. Ferguson & Chapman (86), however, concluded from a detailed study of 16 fatal cases that the syndrome of peripheral vascular collapse and early death in fulminating human meningococcemia is due to injury of many vital areas by the overwhelming infection, rather than primarily to destruction of the adrenal cortex by hemorrhage. The reticulo-endothelial (RE) system seems to be involved in protection against endotoxins of gram negative bacteria. Thomas & Mogabgab (89) found that rabbits treated with ACTH or cortisone developed a local Schwartzman-like reaction to a single intracutaneous injection of meningococcal toxin. Smith & Thomas (90) showed that rabbits were more than 50 times as resistant to the lethal effect of meningococcal toxin at 4 to 7 weeks of age as at 10 to 12 weeks. RE blockade enhanced the susceptibility of both groups and eliminated this difference. Smith, Thomas & Good (91) showed that a single intravenous injection of meningococcal toxin brought on the generalized Schwartzman reaction in rabbits subjected to RE blockade or administration of cortisone. They suggested that the effect of the preparatory injection usually necessary for the Schwartzman reaction is to interfere with detoxification by the RE system. The treatment with cortisone, however, prevented the lethal effect of the toxin (90). The essential role of occlusive thrombosis

of small blood vessels was substantiated by Good & Thomas (92), who showed that parenteral administration of heparin with the provocative dose of meningococcal toxin prevented the development of both dermal and generalized Schwartzman reactions.

Some metabolic effects of meningococcal endotoxin have been reported by Kun *et al.* (93 to 97). Intravenous injection produced a rapid decrease of the histamine in the blood and an increase in the liver and muscle of rabbits. There was an increase in the blood glucose, lactic acid, and inorganic phosphate, followed by a hypoglycemia before death. [An analogous syndrome in man has been reviewed by Fox, Kuzma & Washman (98).] Liver and muscle glycogen decreased, whereas tissue lactic acid increased. Blood and tissue pyruvate decreased. The succinic dehydrogenase activity of muscle and liver was markedly reduced but cytochrome oxidase was not affected. The concept of a tissue anoxia was advanced. *In vitro*, the toxin inhibited the synthesis of glycogen from glucose or pyruvate by slices of rabbit liver. Utilization of glucose by extracts of rat muscle was inhibited. This inhibition was prevented by insulin, indicating that the endotoxin inhibited hexokinase. Recently, Thomas, Smith & Korff (99) reported that a heparin-precipitable protein resembling fibrinogen appeared in the plasma of rabbits following injection of endotoxins from meningococci, *Serratia marcescens*, or *Shigella paradysenteriae*.

Goeters (100) reported that the intracerebral injection of meningococci suspended in egg yolk and broth produced a serially transferable purulent meningitis in mice. Strains of relatively low virulence by the intra-abdominal route gave the best results intracerebrally. Since he found the central nervous system of the mouse to be very insusceptible to meningococcal endotoxin, he concluded that the intracerebral infection measured toxigenicity, whereas the intra-abdominal route measured power of multiplication.

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## EFFECT OF IONIZING RADIATION ON RESISTANCE AND INFECTION

BY DAVID W. TALMAGE

*Department of Medicine, University of Chicago, Chicago, Illinois*

Before embarking on a general review of the effect of radiation on infection and resistance, it is necessary to emphasize the fact that the resistance of a host to infection is the product of a large number of factors which may have varying importance with different infections. Indeed, many factors, such as age (1), state of nutrition (2), stress, and allergic response may have opposite effects in the same host for two different disease agents (3).

Injury from ionizing radiation has many direct and indirect (4) physiological effects, each with its own threshold and dosage response curve, and varying in time for maximum development and recovery [See reviews by Cronkite & Brecker (5); Ord & Stocken (6); and Patt (7)]. Most if not all of these physiological effects of radiation might conceivably alter some host-parasite relationship. Anemia, leucopenia, plasma electrolyte and protein disturbances, ulceration of the gastrointestinal tract, and altered secretion of adrenal corticoids (8, 9, 10) are only a few of the many effects of radiation that probably alter host resistance. It is not surprising that studies of individual host-parasite relationships should show that x-radiation can have a beneficial as well as a deleterious effect upon host resistance, depending on the particular host-parasite relationship studied, the time of irradiation, dose of irradiation, and other factors. Indeed, the same animal may show both the increased and decreased resistance to the same organism at different times following the same dose of radiation. The literature demonstrating these varied effects of radiation has been adequately covered in the review by W. H. and L. G. Taliaferro (11).

Of the numerous factors involved in resistance, those associated with cellular and antibody reactions have the most widespread application and have evoked the most interest. This review will be confined to an attempt to clarify their interrelationships in radiation injury. The impression is sometimes given inadvertently by those working with either cellular or antibody reactions that one particular mechanism is all important in host resistance. The problem is, however, considerably more complex. For some disease agents, e.g. measles, antibody responses appear to be most important; for others leucocytes appear so [See review by Wood (12)]. Some organisms may be destroyed by either leucocytes or lytic antibodies acting alone, while others require the cooperative action of opsonization and phagocytosis.

### ROLE OF INFECTION IN RADIATION DEATH

Animals or men receiving very large doses of ionizing radiation may die from injury to the nervous system or gastrointestinal tract before infection



has had a chance to set in. Evidence that infection plays a role in death from median lethal doses of radiation falls into two general categories.

(a). *Evidence of infection before or at the time of death.*—In Japanese dying after atomic bombing (13) or in irradiated animals (14), obvious infection has been reported in the form of cellulitis, mucosal ulcerations, pneumonia, and septicemia. In mice receiving lethal doses of radiation, the presence of a positive blood culture has been closely correlated with the time of ensuing death [Miller *et al.* (15, 16)]. The difficulty in interpreting these experiments lies in determining whether or not the infection is the cause of death or a manifestation of the agonal state.

(b). *Increased survival in germ-free rats and animals treated with antibiotics.*—Reyniers *et al.* (17) have demonstrated a definite increase in survival time of germ-free rats receiving 400 to 1000 r and an increased absolute survival in a more limited range from 400 to 600 r. Because germ-free experiments are necessarily cumbersome and difficult to control, the use of antibiotics probably represents a more practical approach to the same problem. In a number of different species, the use of one or a mixture of antibiotics has generally but not always resulted in an increase in survival time and a decrease in mortality rate following irradiation within a fairly limited dose range up to LD<sub>90</sub> [See review by Cronkite & Becker (18)].

*Granulocytes.*—The sensitivity to radiation of the various species of animals studied varies considerably when this sensitivity is expressed in terms of survival rate (LD<sub>50</sub>). The LD<sub>50</sub> seems to be well correlated with injury to the hematopoietic tissue of the bone marrow, and more particularly with injury to the granulocytic series. Cronkite & Brecker (18), who have reviewed the evidence for this statement, are investigating the effect of leucocyte transfusions in irradiated animals.

Convincing evidence has been presented by Smith *et al.* that survival of the mouse following radiation is closely correlated with the peripheral granulocyte count (19). This was true in their experiments whether death occurred spontaneously following a high lethal dose of radiation or whether following a sublethal dose of radiation the animal was challenged by a subcutaneous injection of *Pseudomonas aeruginosa* (20). As these authors concede, it is not possible by such experiments to rule out the role of other factors. Shechmeister has pointed out the fact that in addition to the number of granulocytes, their functional capacity to ingest and destroy bacteria may be important (21, 22). Furthermore, this particular species, the mouse, may be unique in the absence of bactericidal antibodies [Marcus *et al.* (23)], and, if formed, it is likely they would be uniformly suppressed below effective levels by radiation. Antibodies are probably like antibiotics which require bactericidal levels to be effective in the absence of leucocytes [Marston *et al.* (24)].

*Radiation protection factor.*—Increased survival and more rapid re-

covery of the hemopoietic tissues of mice, rats, and hamsters following a high lethal dose of radiation has been reported following a number of experimental procedures. These include spleen-shielding, with or without subsequent splenectomy, parabiosis (25), and administration of cells or "cell extracts" from spleen, bone marrow, or mashed embryos. The literature concerning this radiation protection factor has been reviewed recently by Jacobson (26) who cites evidence in favor of the view that there is a humoral factor (or factors) responsible for the regeneration of hematopoietic tissues. This hypothesis is based on histological observations of reticular cell regeneration and the effectiveness of heterologous splenic transplants and cell suspensions (27). Although Jacobson believes that it would be unlikely for heterologous cells to seed and temporarily repopulate the hematopoietic tissues of the radiated recipient, this possibility has not been ruled out. Irradiation of the recipient has been shown to increase the take of grafts of normal homologous tissue (28) and heterologous tumors (29, 30).

Cole & Ellis (31) have reported experiments which they believe show that the radiation protection factor of a mouse spleen resides in the nuclear fraction. As evidence that this factor is noncellular they describe experiments demonstrating the destruction of this factor in spleen nuclei by deoxyribonuclease and trypsin, enzymes thought not to attack living cells. However, treatment with these enzymes destroyed the protection factor equally as well or better when fresh spleen homogenates were used. To follow the same line of reasoning this would indicate that the living cells present in the fresh homogenates contained no protection factor. This conclusion is contrary to the rather general observation that the more handling given the cells in spleen homogenates the less protection factor they will contain. It seems reasonable to say that the exact nature of the radiation protection factor remains unsettled.

Although survival and death are very practical aspects of any study involving radiation, they represent only a small fraction of the total measurable radiation effect. Many of these effects may be profitably isolated and studied exhaustively by techniques with endpoints less complex than death. In the process a great deal may be learned of basic immunological mechanisms. In the following pages, some of the studies involving lymphocytes and antibody responses will be reviewed.

#### LYMPHOCYTES

Following whole body irradiation the lymphocytes in the peripheral blood decrease in numbers more quickly and from a smaller dose of roentgens than the other white cells and require a longer time for recovery (26, 32). The lymphocytes decrease immediately following exposure, and the greatest part of the drop occurs in 18 to 24 hr. A detectable effect occurs following as little as 25 r. The response of the circulating lymphocytes is fairly

comparable in most species which have been studied (32), a striking contrast to variations in the susceptibility and recovery of the granulocytes (18).

Little is known with certainty concerning the function of the circulating lymphocyte; consequently the literature is filled with speculations. Thus to the lymphocyte was attributed first the function of antibody producer (33, 34, 35) and more recently of antibody carrier (36, 37, 38, cf. 39, 40). That it is not a necessary cellular agent for these functions is indicated by the fact that antibody production may continue normally at a time after radiation when the lymphocyte counts are minimum (See 41 and discussion below).

Maximow & Bloom (42) consider the lymphocyte a multipotential cell which may become transformed into inflammatory macrophages or other forms of leucocytes. These conclusions are based on careful histological observations and have been partially supported by Farr (43) using diamino-acridine dye tracers. Others [44; and reviews by Rebeck (45); Taliaferro (46); and Yoffey (47)] have described the importance of these cell transformations in immunity. Against this view of the activities of the lymphocyte may be the failure of lymphocyte transfusions in the irradiated rat (48) or of spleen-shielding during irradiation of the rabbit (49) to increase survival rate or hasten recovery of the granulocytes. These latter experiments are not conclusive, however, since the factors required for the development of the lymphocyte may be absent in the irradiated animal.

A relationship between the lymphocyte and the antibody response is suggested by the close temporal correlation in the irradiated rabbit between the level of the circulating lymphocyte and the ability of the animal to respond to the injection of an antigen. Both the lymphocyte count and the ability to respond to antigen are intact immediately following radiation in the rabbit (41), but drop off rapidly thereafter, reaching minimum levels in 24 to 48 hr. after as little as 300 r. Both require 30 days or longer for full recovery, despite the fact that lymphoid tissues of spleen and lymph node appear fully recovered much earlier. Harris has reported that the ability of an irradiated rabbit to respond to an injection of particulate antigen may be restored temporarily by the simultaneous injection of cells from the lymph node of an unirradiated animal (50). Jaroslow & Taliaferro (51) found a similar effect in the rabbit with homogenates of homologous spleen. Even more striking and difficult to understand is their finding that mouse spleen homogenate, mouse ascites tumor, or Hela tissue culture strain of human carcinoma is also effective. These studies are challenging because of their implications to the basic mechanisms of antibody production.

#### ANTIBODY RESPONSES

*Primary response.*—Perhaps the outstanding impression gained by many early workers was the marked radiosensitivity of the immune response. Re-

ardless of the antigen-antibody system or species studied, marked suppression of the primary antibody response was demonstrated after a relatively small dose of whole body radiation (100 to 200 r) given 24 to 48 hr. before the injection of antigen (For review see 11).

More recently W. H. and L. G. Taliaferro (52) have found that a plot of the log x-ray dose against the log peak antibody titer gives a typical sigmoid dosage response curve. This finding should make it possible to compare this type of injury with median lethal doses and other radiation effects. It may be argued that it was the correlation between the radiosensitivity of certain lymphoid cells and the antibody response which was responsible for the development of the concept that lymphoid tissue was the major source of antibody production (53). This concept has been further strengthened by evidence of antibody production within lymph nodes (54, 55) and by tissue culture cells of the lymphoid series (56). Histological changes indicating protein synthesis have been found in plasma cells to coincide with the time of maximum antibody production (57 to 60). In certain situations splenectomy has been shown to depress antibody formation greatly (61, 62) and cells from spleen (63) and lymph nodes (64) have been found capable of transferring antibody production to other animals.

As convincing as these experiments are in implicating certain lymphoid cells in the antibody response, they should not be construed as indicating that the antibody response is a simple, one-stage, all-or-nothing response involving a single type of cell. On the contrary, there is considerable evidence to indicate that the immune response is a complex and varied phenomenon involving at least two phases and that its susceptibilities to radiation may vary considerably depending on a number of factors including: (a) time of x-irradiation relative to antigen injection; (b) previous exposure to the antigen; and (c) character of the antigen.

*Time of x-radiation relative to antigen injection.*—The earliest workers studying the effect of x-radiation on the immune response noticed that to be most effective in suppressing antibody formation x-radiation must be given before the injection of antigen (65 to 68). Recently, three independent studies were reported by Kohn (69), by Dixon and co-workers (41), and by Taliaferro *et al.* (70), which amplified this observation and cast some light on its significance.

Kohn measured hemolysin titers in rats and found that if x-radiation were given after the injection of antigen or even on the same day, considerably higher doses of radiation were required for suppression of antibody formation than if x-radiation were given before the injection of antigen. He suggested the possibility that the fixation of antigen by antibody-forming sites might be a more radiosensitive process than the later stages of antibody formation. In similar experiments in rabbits Dixon *et al.* used the response to bovine gamma globulin and Taliaferro *et al.* the hemolysin response to

sheep red blood cells. Both these groups studied the time of injection more closely than Kohn and both made the key observation that immediately following x-radiation of an animal there were a number of hours during which an injection of antigen would result in a nearly normal antibody response. Both Dixon and Taliaferro suggested a relatively short radiosensitive initial or "adapting" phase of the antibody response and a later relatively radio-resistant production phase. These results suggest that sublethal doses of x-radiation do not directly destroy the cells involved in antibody synthesis. Recent experiments by the Taliaferros indicate that under certain circumstances, irradiation after the injection of antigen can have a stimulatory effect on antibody formation (71). The ability of an animal to respond to an antigen injected immediately after radiation but not 24 hr. later suggests that radiation damages a precursor of antibody-producing cells. An alternative explanation (which does not exclude the first one) is that cells of the lymphoid series are capable of reacting to antigen at only a certain phase in their development and that x-radiation, without killing the cells, prevents them from developing into this antigen-reactive stage.

*Selective shielding and irradiation of the spleen.*—Experiments involving selective shielding during irradiation or selective irradiation of the spleen before injection of antigen cast further light on the complexity of the immune response. Jacobson (72, 73), Wissler (74), and their co-workers have pioneered in this field, especially with the rabbit and the rat. Of the two animals the rat showed the most protection of the immune response in the spleen-shielded animal when the antigen was injected intravenously. In the rabbit the protection afforded by spleen-shielding during irradiation was much less than might be expected by the fact that in unirradiated animals given the same injection of antigen splenectomy has been shown to reduce antibody production markedly (61, 62). Splenectomy 24 hr. after radiation of a spleen-shielded animal (and 24 hr. before injection of antigen) did not reduce the resulting titer significantly below that obtained by spleen-shielding alone (75). This would seem to indicate that, even in a situation where the largest fraction of antibody is produced in the spleen, protective effects obtained by spleen-shielding during irradiation can better be explained by protection of non-splenic antibody production than by a protection of splenic antibody. Taliaferro has made similar studies in rabbits using a more quantitative method of measuring hemolysin titers to sheep red blood cells, the 50 per cent hemolytic unit (76). Animals spleen-shielded during irradiation showed titers of less than one-third that of unirradiated controls. Conversely, animals given large amounts of irradiation (up to 1400 r) selectively to the spleen produced normal or above normal amounts of antibody. Do these experiments indicate that the antibody production in one site depends on cells obtained from other parts of the body? This conclusion seems to be borne out by the experiments of Harris showing that an irradiated rabbit can

respond to an injection of antigen if it receives at the same time an injection of cells from the lymph node of an unirradiated animal (50).

*Previous exposure to the antigen.*—Useful as the preceding experiments have been in the development of concepts of antibody formation, it is doubtful that the radiosensitivity of the primary antibody response has much direct bearing on the general problem of post-irradiation resistance to infection. This is because it is highly unlikely that, within the limited period of maximum radiation effect, an animal would be exposed to an organism with which it has had no previous contact. When one considers the large elements of cross reactivity existing between different species of bacteria, the primary immune response becomes even less a factor in host resistance.

Both Dixon *et al.* (41) and Taliaferro *et al.* (70, 71) studied the specific anamnestic response after total body x-radiation. Using the same techniques which had yielded essentially identical results with the primary response, these two groups obtained completely opposite results with the secondary response. Dixon *et al.* found the secondary response to bovine gamma globulin after x-irradiation was more prompt than the primary response in the unirradiated control and only slightly delayed compared to the secondary response in unirradiated controls. The suppression of the secondary response after 800 r was minimal compared to a complete suppression of the primary response by 400 r [For confirmation see Silverman & Chin (77)]. Taliaferro *et al.* found the secondary hemolytic response to sheep red cells to be equally as sensitive to radiation as the primary response. These conflicting results may be explained by differences in the nature of antigen used or in the type of antibody formed. Marcus reported that disintegration of erythrocytes (78) and bacteria (79) within macrophages is inhibited by x-radiation, whereas Dixon *et al.* (80) found no difference in the non-immune metabolism of bovine gamma globulin after x-radiation. This would suggest that x-radiation inhibits a preliminary digestion of one antigen but not of the other. On the other hand, the finding that the hemolytic and precipitating antibodies differ in molecular size (81) and rate of metabolism (82) suggests that these may represent two fundamentally different types of antibody response. Since the character of the antigen may well govern the type of antibody response these two explanations possibly represent two sides of the same phenomenon. Regardless of whether either or both represent the correct mechanism, it is apparent that resistance to infection following radiation depends, among other things, on the susceptibility of the secondary immune response to the particular disease agent involved.

Perhaps even more important than the susceptibility of secondary response to irradiation is the radiosensitivity of the antibody response to low-grade continuous antigenic stimulation.

Miller and co-workers (14) have shown that infection occurring after x-radiation of mice is generally caused by organisms which naturally inhabit

the intestinal tract. A relative immunologic refractoriness (83) or unresponsiveness (84) has been shown to occur after long, continuous antigenic stimulation such as might be produced by the intestinal flora. The effect of x-radiation on the production of antibodies under these circumstances has yet to be studied. However, the so-called "natural" antibodies provide a field for conjecture in this regard since it has been postulated that these antibodies, which occur in low titer, are the result of continuous antigenic stimulation from the intestinal bacteria or from antigens present in the ingested food [Wiener (85)].

Marcus & Donaldson found that the level of "natural" bacteriolysin was low in rabbits 7 to 15 days after 650 r total body irradiation, but not after 500 r (86). The levels of "natural" agglutinin and complement were unaffected by either dose of x-radiation (87). The disappearance of "properdin" (88) from the blood of rats following 500 r total body x-radiation is probably a similar phenomenon. "Properdin" or antizymosan should be included in the group of "natural" antibodies until more is known about the latter's source and character. It is possible that there are other antibodies which, like properdin, require the presence of complement to combine with antigen.

*Disappearance of pre-formed antibody.*—Following x-radiation, an animal's ability to form new antibody may or may not be impaired, depending on the circumstances as discussed above. An inability to form new antibody will not necessarily have a fatal effect since the animal possesses at the time of irradiation an adequate amount of "natural" or immune antibody to protect him. Although x-radiation does not alter the rate at which this pre-formed antibody is destroyed (89, 90), there is a large variation between species in the turnover rate of serum globulins (91). A comparison of the rate of disappearance of antibody (91) and the time of recovery from the radiation effect (18) in various species reveals the striking fact that there is a greater variation in rates of antibody metabolism than in the time required for recovery. To illustrate the effect of this difference consider the situation that obtains in mouse and man. The half disappearance time of antibody in man has been estimated at 14 to 21 days, and average time for recovery from a single mid-lethal radiation exposure as 63 days. By the time man recovers from x-radiation he would suffer only an 8- to 16-fold drop in the level of antibody present prior to radiation. On the other hand, the half disappearance time of antibody in a mouse has been found to be 1 to 2 days and recovery time, 30 days. The mouse would have, by the time of recovery, a drop in pre-formed antibody amounting to a factor of  $10^6$  to  $10^7$ . If one takes instead of time to recovery, the time to maximum radiation effect or minimum white blood count, the differences are equally striking.

In addition to differences between species in rates of antibody metabolism, recent evidence obtained by Taliaferro *et al.* indicates that, in the same species, there is a difference between antibody types in rate of disappearance



(82). In this instance they found a half disappearance time of slightly less than 3 days for hemolysin in the rabbit, which should be compared to a half disappearance time for rabbit precipitin of 5 to 6 days (91). Marcus' studies with bacteriolysins in rabbits (86) showed a better than 10-fold drop in 9 days after x-irradiation. Even assuming no additional production of antibody, these figures would indicate a half disappearance time of less than 3 days. Pillemers' results with properdin in the rat similarly indicate a half-disappearance time of 1 day (88). It is important to determine whether this rapid disappearance rate may be due in part to unusual absorption of antibody by bacteria and whether it applies generally to bacteriolytic antibodies. If so, then the irradiated animal, depleted of leucocytes, may also be unhappily deprived rapidly of the type of antibody capable of destroying bacteria unaided by cells.

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## IMMUNIZATION<sup>1</sup>

BY GEOFFREY EDSALL, M.D.

*Immunology Division, Army Medical Service Graduate School,  
Walter Reed Army Medical Center, Washington 12, D. C.*

Immunization is but one facet of the larger complex of phenomena comprised by infection, resistance, and the related fields of blood and tissue immunology, the hypersensitive states, etc. It is impossible to separate the subject of immunization, let alone a portion of it such as will be dealt with here, from the whole without accepting certain limitations to the scope of the discussion. This review is concerned with certain aspects of active immunization against infectious diseases, which have developed either in clarity or in complexity during the past five years, and which serve to illustrate principles or problems which apply to the subject as a whole. Specific disease entities will not be dealt with separately, but the review will be concerned with various aspects of the immune response and the factors which influence its pattern; with tolerance of antigens; with certain host factors affecting immunity; with antigenic competition; and with the rapid development of resistance. Various topics related to the subject at hand have been competently considered, in various volumes of this series: e.g., by Freund chiefly on adjuvants (1), by Gowen on inheritance of immunity (2), by Raffel on types of acquired immunity (3), by Taliaferro on the cellular basis of immunity (4), by Clark on nutrition and resistance (5), by Maaløe & Jerne on biologic standardization (6), by Sigel on age and resistance (7), by Kass & Finland on endocrine factors (8), by Haurowitz on the immunological response (9), by Hilleman on influenza virus (10), by Coons on labelled antigens and antibodies (11), by Nungester on non-specific factors (12), and by Oakley on toxins (13). Also, much general information pertinent to the topic of this review was brought together four years ago in a symposium edited by Pappenheimer (14).

Artificial active immunization is, basically, an attempt to get something for nothing. Even variolation against smallpox—a barbarous practice by present standards—was eagerly sought by thousands in the eighteenth century, for it offered only about a two to three per cent chance of death as against the 15 to 25 per cent chance that went with smallpox contracted naturally. However, since Jenner followed the impulse provided by Hunter's famous remark,<sup>2</sup> and Pasteur, three-quarters of a century later, applied

<sup>1</sup> The survey of literature pertaining to the review was completed in December, 1954, but a few more recent papers have been cited.

<sup>2</sup> Usually, but perhaps incorrectly cited as "Don't think; try it!"

the principle of attenuation in rational fashion to fowl cholera and anthrax, attempts have been made to immunize artificially against almost all proved (and a good many unproved) infectious diseases of man and animals. Yet one can count on the fingers of both hands the number of infectious diseases of man for which active immunization has clearly been shown to be of more than marginal effectiveness as a prophylactic procedure; and the list for animal diseases is not much longer. On the other hand, there is a large list of infectious diseases, for which immunization appears to be of some value, but for which the evidence is subject to many reservations and doubts. Obviously, certain conditions must be met if artificial immunization is to succeed in any given instance. There must be, in some degree at least, a recognizable pattern of naturally acquired immunity to the disease; the virtual absence of such a pattern is sufficient reason for lack of any immunization procedure against, say, malaria or amebic dysentery. Also, it must be possible to prepare an antigenic substance or mixture of substances from the infective agent (e.g., pertussis) or from an antigenically related agent (e.g., smallpox or yellow fever) in sufficient quantity to be applicable to the problem. This preparation must be safe enough to be used in man under present day standards of caution, and capable of inducing at least a recognizable immune response, lasting long enough to be of some practical value. These are the minimal requirements. In the case of diphtheria and tetanus they have been readily met, since the weight of evidence regarding the pathogenesis of each of these diseases indicates that immunity to their respective toxins is the essential prerequisite to protection against the disease. Success in these two diseases has been tremendously facilitated not only by the simple nature of their pathogenesis, but by the fortunate finding that it was chemically possible to abolish the toxicity of the toxins without significantly reducing their antigenicity; and by the further finding that the response of man to these detoxified toxins or "toxoids," properly administered and scheduled, is vigorous and lasting, as will be noted below. Such good fortune has not attended the efforts to immunize against most other diseases. However, it is interesting to note that more convincing, statistically significant evidence has been accumulated for the efficacy of such bacterial vaccines as, for example, the BCG strain of *Mycobacterium tuberculosis*, or pertussis vaccine, than it has been possible to collect even for diphtheria or tetanus toxoids. This is not said to imply that the toxoids are not highly effective, but rather to point out that where widespread skepticism has existed, it has been possible to undertake carefully organized and adequately controlled field trials; and, by means of these trials, statistically significant answers can be obtained, at least for the circumstances under which the trial was conducted. Aside from several studies of this type employing BCG vaccine, carried out some years ago and previously reviewed by the writer (15), additional data such as those published recently by Aronson

(16) provide evidence for the biological effectiveness of this vaccine. It is not within the scope of this short review to consider the practical value of BCG vaccine, which involves many considerations not germane to the fundamental scientific question. As regards pertussis vaccine, the lingering doubts reflected in previous reviews (15, 17), which persisted despite the accumulation of more controlled data than have ever been collected for any other immunizing procedure, have perhaps been at last laid to rest by the incomparable field trials carried out under the auspices of the British Medical Research Council (18). No controlled studies of this scope and statistical conclusiveness have ever been carried out for any other immunizing procedure.<sup>3</sup> It is obvious that no such studies ever will be carried out for the evaluation of immunization against smallpox, yellow fever, diphtheria, or tetanus, either because they would be epidemiologically impossible or because the existing data provide such overwhelming evidence for their value that a controlled study would appear not only unnecessary but inhuman. However, for such vaccines as those for typhoid fever, cholera, and plague it may be hoped that soundly conceived and rigidly controlled field studies may some day settle, once and for all, the role of these agents in the effective control of infectious diseases in man.

#### PATTERN OF THE IMMUNE RESPONSE

Assuming that whatever protection is achieved can be related to a measurable systemic immune response, much interest has naturally focussed on the factors which influence the degree and duration of the antibody response to a given antigen. Many of the factors affecting the immune response have been discussed by Dixon *et al.* (19), Edsall (20), Heidelberger (21), and Freund (22). Certain additional findings, however, deserve mention here.

*Amount of antigen used.*—Bousfield & Holt (23) have explored the relation between dosage of aluminum-phosphate adsorbed diphtheria toxoid, from 0.5 to 30 Lf units, and the per cent of Schick conversions induced in non-immune children. Plotting their results on a probit basis, they find a straight-line relationship up to a dose of about 2.5 Lf units, above which the degree of response levels off—due in part at least to the fact that it is impossible

<sup>3</sup> The number of subjects included in the vaccine-placebo phase of the superbly executed 1954 Poliomyelitis vaccine evaluation (18a) was many times larger (401,965) than the number in The Medical Research Council pertussis vaccine study (6,710) or any other controlled study ever before undertaken; however, the number of cases on which to base an analysis of the poliomyelitis study was considerably less (244 vs. 836)—a comparison which vividly reflects the difficulties involved in studying such a relatively rare disease as clinical poliomyelitis.



to measure more than 100 per cent Schick conversion. Edsall, Banton & Wheeler (24) found that, in medical students, using doses of diphtheria toxoid ranging from 0.0016 Lf to 1 Lf, a straight-line relationship between dose and response appeared in a log-log plot, with a slope of close to 0.5. They noted that the findings of various other investigators, employing tetanus toxoid or influenza vaccine, yielded similar slopes. This slope is, of course, as yet not only approximate but empirical; it simply provides a basis for further investigations with other antigens and other methods of measurement, in man and other species. However, it gives a useful tentative working concept of the results that may be expected from varying the dose of an antigen, and suggests that rigid adherence to maximal dosage in immunization procedures may not—at least at the booster phase—be as imperative as is sometimes assumed.

The importance of dose may also be surmised, though not conclusively proven, in the various reported observations on the effect of antibiotics on the immune response in infectious diseases. Stevens (25) has summarized and discussed a number of such reports, in each of which it may reasonably be assumed that the early application of an antibiotic curtailed the production of antigen and hence impaired the immunological response to the infection. A more quantitative approach to this concept has been provided by Smadel (26) who described the antibody response and the frequency of relapse in natural and experimentally induced scrub typhus treated with chloramphenicol at various intervals after the establishment of infection. Treatment of the naturally occurring case, which ordinarily was not instituted until the eighth day or later, resulted in rapid cure, no relapses, and an early and steadily rising Weil-Felix reaction. Treatment of infected volunteers, begun on the sixth day, was followed by 37 per cent relapses, although no measurable difference in the Weil-Felix response was found. Treatment on the third day of illness was followed by 62 per cent relapses, and a marked delay in the appearance of OX-K agglutinins. An even more striking correlation was noted in volunteers receiving living Karp strain scrub typhus vaccine, with or without antibiotic prophylaxis. Smadel concludes that "these responses in scrub typhus would appear to depend upon the quantity of rickettsial antigen which becomes available for stimulating antibodies."

*Pattern of the immune response.*—The outline and examples furnished in Chapter III of Burnet & Fenner's monograph (27) still stand as the simplest and yet the most adequate description of the basic characteristics of the immune response pattern. Dixon and his co-workers have compared the pattern they observed following the injection of bovine serum albumin or bovine serum globulin, with the patterns described by others who used erythrocytes, toxoids, or bacterial polysaccharides (28). Their comparison, slightly modified and adapted to tabular form, may be summarized as follows:

Characteristic	Erythrocytes	Toxoid	Polysaccharide	Bovine serum proteins
Appearance of antibody	3-4 days	2-4 weeks	2-6 weeks	6-9 days
Antibody peak	3-6 days	2-3 months	1-2 months	early
Antibody decline	first rapid then slow	first rapid then slow	slow	first rapid then slow
Booster rise	2-3 days	2-3 days	slight or absent	3-4 days
Booster decline	rapid	first rapid then slow	slow	intermediate
Ratio, 2ndry/1ry response	1:1	10-100:1	1:10 $\pm$	10:1
Persistence of Antigen	brief?	brief?	prolonged	?

However, such comparisons are not entirely justified, since the experiments were done by different workers under different conditions. For example, Dixon's antigens were given in doses of 30 mg. per kg. of rabbit. This is the equivalent in weight of 10,000 Lf diphtheria toxoid per kg., a dose of this antigen which, to the best of this reviewer's knowledge, has never been studied. Should such doses be employed, it might well be that the appearance of antitoxin might be detected much earlier.

The secondary response has been analyzed by Holt, working with aluminum-phosphate precipitated purified diphtheria toxoid ("P.T.A.P.") in guinea pigs. Holt (29) describes three components: (a) the pre-booster titer, (b) the "pure" second response effect or "leap," and (c) degradation from the new maximum to a baseline. If the contribution of the initial primary antibody-forming mechanism is deducted, the degradation appears to be a first order process. However, it is hardly possible to generalize with this degree of precision regarding the secondary response. Barr & Llewellyn-Jones (30) quite reasonably point out that such a concept would not adequately account for the wide variation in the degradation patterns observed after a booster response, for the sometimes enormous scale of the response, even in animals or humans with no detectable pre-booster antibodies, or for the delayed or sustained response described by Barr & Glenny (31), which has been given inadequate consideration in interpreting the long-term effects of immunization.

*Adjuvants.*—Freund (32) published an excellent review of the available knowledge regarding the phenomena associated with the use of water-in-oil emulsions as antigenic adjuvants, and relatively little of fundamental importance has been added since then, as far as the mechanism of their action or the response that such adjuvants induce is concerned. Talmage *et al.*

(33), using a radioactive tag, traced the disappearance from the injection site of antigen in saline, in alum, or in Freund's emulsion. The rate of disappearance of antigen in alum was almost as rapid as that in saline, whereas antigen in water-in-oil emulsion disappeared very much more slowly. Freund *et al.* (34) have found that the concentration of antibody around a water-in-oil injection site is considerably higher than in the blood, whereas at a control site where broth was substituted for antigen the reverse was the case. Salk, Bailey & Laurent (35) suggest that at the site of inoculation of a water-in-oil emulsion of antigen there is an accumulation of "cells important in antibody formation. The antigen which is retained at the site of inoculation is thereby brought into intimate contact with the antibody-producing cells rather than being dissipated throughout the body where contact with antibody-forming cells is effected much less efficiently." This is a rational working concept but the actual situation is probably not quite as simple as this implies. In the first place Freund (36) has found that the effectiveness of a water-in-oil emulsion is only moderately reduced if the inoculation site is extirpated as early as  $\frac{1}{2}$  hr. after injection. These findings are hard to reconcile with those of Talmage *et al.* cited above, but it may be reasonable to suppose that (a) at least a small amount of antigen in the Freund emulsion is rapidly lost from the injection site, probably along lymphatic channels very soon after injection, probably being caught up in the regional lymphatics, and (b) since the dose response relationship has such a flat slope, as noted above, it will necessarily be difficult to distinguish significant differences in antibody levels resulting from anything less than extreme differences in effective dose of antigen.

Recently, Wood (37) has reported that, using either C-reactive protein or human gamma globulin as antigens administered intravenously in rabbits, markedly increased precipitin titers were obtained if a form of the "Freund adjuvant" (in this case a stable emulsion of mineral oil with "Aquaphore" in saline) was injected subcutaneously into the animals at the start of each course of antigen injections. (It is not clear whether, in the experiments demonstrating this increase in antibody formation, tubercle bacilli were incorporated into the adjuvant or not.) Quantitative precipitin values are not given, and it is therefore not feasible to compare the results obtained by Wood with findings such as those presented by Freund and many others, who have incorporated the antigen in the water-in-oil emulsion. A possibly similar type of adjuvant effect, induced by typhoid endotoxin, has been briefly reported by Johnson *et al.* (38). Both findings are extremely interesting and may prove highly significant, but they are as yet insufficiently studied to be well understood or to be compared satisfactorily with either the classical adjuvant mechanisms referred to above, or with the numerous reports of nonspecific simultaneous adjuvant actions ascribed to a great variety of substances by various investigators over the past 50 years, and generally regarded as aspects of the nonspecific anamnestic response.

*Duration of immunity.*—Naturally acquired immunity, either actual or

potential, can persist at least 60 years, as was found by Panum over a century ago, in his classical studies of measles in the Faroe Islands. MacLeod (39) has assembled evidence for the postulate that such long-lasting immunity is due not so much to the persistence of measurable antibody, as it is to the retention of the capacity to respond to a "booster" experience on re-exposure to the infectious agent. This postulate in turn requires the assumption that lasting immunity be correlated with a relatively long incubation period, to allow time for the booster mechanism to work. However, this concept does not account for all known situations, since antibodies to the yellow fever virus have been found in individuals as much as 50 years after recovery from the disease; and even after the relatively weak stimulus of vaccination with the 17D strain of yellow fever virus, protective antibodies were found in 77 per cent of vaccinated subjects nine years after vaccination (40). A similar long persistence of detectable antibodies has been found in studies on the persistence of immunity to tetanus in veterans of World War II. Looney *et al.* (41), Turner *et al.* (42), and Peterson *et al.* (43) have all observed unexpectedly high antitoxin levels, in a large percentage of subjects, as late as 9 to 11 years after the last booster dose of tetanus toxoid. Four out of 19 such subjects in Turner's group had over one unit of antitoxin when tested prior to a booster injection, and Looney's subjects showed a similar high maintenance level up to eight years after the last booster. It is of passing interest that similar studies in children by Bigler (44) did not appear to disclose anything like such high persisting levels. Such a difference might be due to the administration of more frequent booster injections to service personnel (in some theatres during World War II, for example, the policy called for booster doses every three months), to an adjuvant effect resulting from the multiple simultaneous inoculations characteristic of military immunization, to a difference in the responsiveness of adults vs. children, or to some unknown factor. The answer must for the present remain a matter of intriguing speculation, since a definitive study on this point would be tedious, complicated, and hard to set up. Nevertheless further information on the point would be extremely valuable.

*Persistence of the antigen.*—The relation of the duration of immunity to the persistence of antigen has been extensively discussed in the review by Coons on "Labelled Antigens and Antibodies" (11) earlier in this series. It is worth noting from the data summarized in his review that it is not only polysaccharide antigens that remain detectable in tissues for long periods, but protein antigens as well, McMaster & Kruse (45) having found conjugated protein and native bovine gamma globulin in the liver and lymph nodes of mice for as long as 120 days—a long period for this short-lived and metabolically hyperactive animal. Furthermore, there is a growing body of evidence that certain antigens—notably the rickettsiae—may persist in the viable state in the host for long periods. This concept was indeed the basis for Zinsser's hypothesis that Brill's Disease was actually a recrudescence of epidemic typhus fever. It has been supported by the isolation of viable

rickettsiae of Rocky Mountain Spotted Fever and of Scrub typhus from patients as long as 12 months after recovery from the disease [Parker *et al.* (46); Smadel *et al.* (47)].

Heidelberger has suggested that the sustained antibody plateau observed after the administration of pneumococcus polysaccharide antigens (21)—and also, as it now appears, occurring after injection of the purified polysaccharide Vi antigen of *Salmonella typhosa* [Landy (48)]—may be due to the absence, in the mammalian host, of enzymes effective in splitting polysaccharides of bacterial origin. Thus it is postulated that the carbohydrate antigen is able to persist in the tissues of the host for longer periods than are available to protein antigens, and, in persisting, to continue to provide the stimulus—template or whatever it may be—for antibody formation.

From the teleological standpoint, persistence of antigen does not necessarily serve the interest of the host. The "paralyzing" effect of large doses of pneumococcus polysaccharides in immunization of mice was noted some years ago and was extensively studied by Felton (49). It has not been clearly demonstrated to occur in other species, although Morgan *et al.* (50) have postulated that it is responsible for the difficulty hitherto experienced in obtaining an antibody response to polysaccharide antigens in rabbits; thus it seems reasonable to suppose that it may be somewhat less rare a phenomenon than has appeared to be the case heretofore. Furthermore, it appears clear that the "paralyzing" effect can be relatively transitory; Baer *et al.* (51) have described its appearance and disappearance over a three months' period.

The persistence of live antigens such as the rickettsiae is likewise of doubtful benefit, as is implicit in Zinsser's concept of Brill's Disease. In confirmation of this concept, it should be noted that not only have Murray & Snyder isolated typhus rickettsiae from cases of frank Brill's Disease (52), but Murray and another group of co-workers (53) have provided strong evidence for concluding that many cases of mild acute illness in such typhus-endemic areas as Yugoslavia are actually cases of recrudescant typhus fever. Such findings, of course, raise more questions than they answer: let us assume that the rickettsiae have a biological relationship to their host fundamentally like that of, say, herpes simplex. Can we undertake to explain the long persistence of immunity to measles on the same basis in view of the generalized susceptibility that Panum found in persons never previously known to have been exposed, and the fact that "recrudescant measles" is not known to occur? Indeed, it is apparent that there is still much to be learned not only regarding the extent and significance of long-persistent antigen, but also as regards the mechanisms underlying long-persistent immunity.

*The booster response.*—Aside from the observations of Holt, Barr, Dixon and others noted above, with reference to the pattern of the booster response, the duration of the capacity to respond to a booster dose has been actively

investigated. The studies on tetanus antitoxin persistence mentioned above (41 to 44) were each also concerned with the response to a booster dose of toxoid. Each study reported an apparently unimpaired capacity to respond to a booster dose for as long as 9 to 11 years. With a completely different antigenic system, Murray, Ofstrock & Snyder (54) and Gauld & Goodner (55) obtained maximum antibody responses to yolk-sac-type typhus vaccine, three to six years after the last previous injection of the antigen. It was of interest that Murray *et al.* (54) found that a second booster injection, either one or seven weeks after the first, induced no additional rise in antibodies—a finding reminiscent of the response of rabbits to another rickettsial antigen (Q fever) described and charted by Burnet & Fenner (27), and of course to the pattern seen with polysaccharide antigens (21). However, before concluding that this type of response is basically different from that obtained with, say, a toxoid, it would be essential to compare the two types of antigens with methods of equal sensitivity in detecting antibodies, and in groups whose titers could be regarded as maximal with equal confidence. Moreover, in interpreting the available data, it should be borne in mind that a great many studies of influenza immunization, and more recent studies on diphtheria immunization [e.g. Edsall, Altman & Gaspar (56); Ipsen (57)] have presented data showing that the relative or fold-increase (although not necessarily the actual quantitative increase) in antibody level is proportionately less as the pre-booster titer is higher. Various examples from other types of study could also be cited, and all these studies suggest that, as ordinarily charted, there appears to be a progressive decrease in the relative antibody response obtainable as the pre-booster level under study is raised. Until comparative studies of the type suggested above are undertaken, therefore, it will be hazardous to state that the booster principle is inoperative in certain immunological systems. It may well turn out that much of the apparent lack of response to a booster inoculation in such studies is due either to the technique of measuring the response or to the logarithmic method of expressing it.

#### TOLERANCE OF ANTIGENS

Starting from the concept set forth by Burnet & Fenner (27) that the ability of the animal host to form antibodies is based upon a capacity to distinguish between "self" and "not-self" characteristics in antigens (i.e., the animal's own tissue is "self" in nature while that of any foreign source is "not-self"). Medawar and his associates have designed a series of experiments to test this hypothesis in its possible relation to the immunology of homografts. In one such study (58) they demonstrated that minced tissue from a heterologous mouse strain introduced parenterally early in the embryonic development of a mouse, established a tolerance to subsequent inoculations of the heterologous tissue. This abnormal tolerance was associated with the absence of any detectable antibody response upon the later introduction of the heterologous tissue, but it could be abolished by passive

immunization against the heterologous tissues. Hanan & Oyama (59), pursuing this concept along other lines, found that introduction of bovine serum albumin into newborn rabbits was followed by inability of these rabbits to form antibodies against this antigen later in life. It has been suggested by Burnet (60) that the apparent absence of an antibody response in man to the virus of serum hepatitis may conceivably be due to some such mechanism as this: if the virus is sufficiently universally carried in man, it may invade the human embryo early in pregnancy, establish itself as a "self"-type substance, and thereby block the subsequent development of antibody against itself in such pre-conditioned individuals. Admittedly, this is a profoundly speculative concept, but it deserves further extension as a hypothetical basis on which to build solid experimental data. In this connection, Dunsford *et al.* (61) have reported a "Blood Group Chimera" in a woman whose blood appeared to be a mixture of groups A & O. The laboratory prediction that she had a fraternal twin proved on inquiry to be correct. Furthermore, Dixon & Maurer (62) have very recently reported findings basically like those of Hanan & Oyama. Therefore, it is no longer possible to doubt that the development of antigenic tolerance in the very young animal can occur. Assuming, however, that a mechanism such as Burnet has postulated for tolerance to serum hepatitis virus exists, corollary problems arise, e.g.: Is this phenomenon more general? Does it play a role, for example, in susceptibility and resistance to herpes simplex? Does it have any bearing on the transient resistance of human beings to the common cold? Many other similar questions will occur to the reader.

#### LIVING VERSUS INACTIVATED ANTIGENS

The first effective immunizing agents were attenuated strains of the infective agent in question. The obvious advantages of using a living agent are: (a) Multiplication of the agent in the host presumably provides a much more extensive immunological stimulus than can be provided by a fixed quantity of a killed agent, and (b) retention of the complete antigenic characteristics of the organism would seem more likely of success in the living state than in a chemically or physically inactivated preparation. The variety of living antigens employed, experimentally or otherwise, is considerable, both in human and in animal immunization; the field has been treated to a lively review by Cox (63). The durability of effective yellow fever and measles immunity (in the absence of repeated stimuli) has been noted above, and typifies the pattern that many investigators feel can be achieved only by the use of live antigens. However, the extraordinary duration of antibody responses potentiated by the use of the Freund adjuvant, as shown by Freund (64) and as may be predicted from the preliminary findings of Salk (65) on influenza, along with such findings as those for tetanus already mentioned (41 to 44), suggest that the heretofore widely accepted necessity for periodic booster doses of inactivated antigens may be subject to revision as techniques of immunization are improved. Meanwhile, the search for safe,



effective, and practical live immunizing agents continues along many lines. Space does not permit discussion of the highly interesting and significant recent developments in this respect regarding such diseases as tuberculosis, poliomyelitis, typhus, and rabies.

Nevertheless, the search for a live antigen which is safe to use in man can be a tedious and often blind and fruitless one.<sup>4</sup> A new approach has been explored independently by Olitzki & Szenberg (66) and by Herzberg *et al.* (67, 68). Both groups conceived and experimentally applied the idea that a living vaccine composed of a streptomycin-dependent mutant of *Brucella* would have the advantages of antigenic integrity characteristic of a live agent, yet would be unable to multiply in the host. Obviously the problem is not so simple as this; back-mutation might occur, or the host might be so unfortunate as to be given streptomycin just after being vaccinated. But these are technical details that can presumably be surmounted, and the principle opens up wide possibilities of application in infections for which current methods of immunization do not appear to be as effective as might be expected.<sup>5</sup>

<sup>4</sup> It may be appropriate here to make some comment upon the difficulties involved in preparing an inactivated vaccine which is safe to use in man. The history of diphtheria immunization, for example, includes several incidents in which toxic products were unintentionally made available for human use, because of inadequate safety testing or sheer ignorance of the principles involved. The Hygienic Laboratory (now the Laboratory of Biologics Control) of the U. S. Public Health Service came into existence largely to serve in the prevention of such hazards; by maintaining through the years a remarkable degree of freedom from political or public pressure, it has set a standard of safety and quality unequalled anywhere in the world. However, new or experimental products continue from time to time to present unforeseen problems. Last year a paper by Sutton & Brooke (65a) reported cases of Venezuelan encephalomyelitis in laboratory workers or maintenance personnel following administration of a formalin-treated chick-embryo vaccine (65b), containing this virus. The most significant aspect of this episode was that no animal test of any sort (and a fantastic number and variety of attempts have been made) had, or has yet, demonstrated the presence of live virus in this vaccine. Yet approximately one human being in every 25 inoculated with the vaccine has become infected with the virus.

One may consider the poliomyelitis vaccine experience of April-May 1955 in the sober light of this little-noticed finding with the Venezuelan encephalomyelitis vaccine. At the time this note is written it is too early to tell the final story of the poliomyelitis vaccine problem; but it is safe to predict that from time to time, man may again prove to be an exceptionally sensitive test animal for the detection of traces of viable microbes in a supposedly "inactivated" vaccine.

<sup>5</sup> A very recent report by Herzberg & Elberg (68a) indicates that, at least in mice, the use of a living streptomycin-dependent *Brucella* for vaccination is no more effective than the same organism killed with phenol, formalin, or heat. However, this detailed observation does not rule out the possibility that living organisms such as these may contain additional antigens, essential in immunization of man or larger animals, which cannot be obtained in an effective form or quantity in inactivated organisms. An additional variation to this principle is the use of a live, virulent agent

## ANTIGENIC COMPETITION

It has been widely claimed for many years—with occasional instances of experimental proof to substantiate the claim—that two or more antigens, given simultaneously, would under certain circumstances interfere with each other. Some years ago Bjørneboe (69) put this concept to a critical test with combinations of pneumococci injected into rabbits. A slight reduction in the antibody response appeared when four or five antigens were given simultaneously, and a marked reduction was seen only with the use of six or eight—hardly an alarming set of findings as far as man was concerned, especially in view of the relatively small quantities of antigen normally used in man. Almost ten years went by during which numerous controlled and uncontrolled studies reported on the advantages of combined antigens [see, for example, reviews by Fleming (70) and Edsall (15)]. Then in 1952 Barr & Glennly (71) reported, in a study of various factors which influenced the antibody levels attained in horses under immunization, that the introduction of a new antigen sometimes depressed the response to an antigen with which the animal had already been immunized. Conversely, the introduction of a second antigen during a period of active response to a preceding antigen might result in a relatively poor response to the second. Following up this lead with better controlled experimental studies, Barr & Llewellyn-Jones (72) showed that (a) pre-existing immunity to diphtheria in guinea pigs appeared to interfere with the subsequent response to the tetanus component of a combined diphtheria-tetanus prophylactic; (b) highly variable results appeared when typhoid-paratyphoid vaccine was substituted for diphtheria toxoid as the competitive antigen. Using several batches of TAB vaccine and various doses and schedules, one batch had no effect at either a high or a low-dose level, one inhibited at the higher level and not at the lower, whereas a third batch had no effect at the higher dose level but was synergic at the lower level. A subsequent study, involving more animals and covering the possible variables more thoroughly, confirmed—in guinea pigs and under the conditions of the experiments—the finding that:

interference with the development of immunity to tetanus toxoid occurred in animals immunized with two injections of a combined prophylactic containing another antigen to which they had some immunity. Similar interference occurred when the first injection was of the combined prophylactic and the second injection of tetanus toxoid alone, but not when the order of these injections was reversed (73).

They concluded that the interference was due to a "crowding-out" effect from the secondary response to the previously employed antigen, whether this was TAB or diphtheria toxoid.

Such observations have far-reaching implications, and a few attempts have been made to confirm or delimit them. Levine & Stone (74) were unable to elicit any interference with primary tetanus toxoid immunization by

for immunization, in conjunction with judiciously timed chemoprophylaxis. Smadel has employed such a procedure in experimental immunization of man against Scrub typhus (26).

simultaneous primary diphtheria toxoid immunization at various dosage levels in guinea pigs; with pertussis vaccine they were able to find only the familiar adjuvant effect. Because Edsall, Altman & Gaspar (56), employing a combined tetanus-diphtheria toxoid containing only 1 Lf of diphtheria toxoid, did not perform tetanus antitoxin titrations, a similar series of injections in Air Force personnel has been studied for antitoxin responses (75). Preliminary results indicate that both diphtheria and tetanus antibody levels, tested just before and three weeks after a six months' "third dose" or booster, exhibited an average and a range not significantly different, in any respect, from similar findings using the two antigens separately. In a better-controlled study, Ipsen (76) has injected two groups of men with fluid tetanus-diphtheria toxoid; one group had received previous tetanus toxoid immunization, the other had not. Slightly but not significantly higher diphtheria antitoxin levels appeared in the group with pre-existing tetanus immunity. Meanwhile reports continue to appear, from time to time, of the "excellent" responses observed in infants or children receiving combined diphtheria-tetanus-pertussis immunization [Sauer & Tucker (77, 78); McComb & Trafton (79); di Sant'Agnese (80); Volk *et al.* (81)]. Since, however, such studies usually report merely the number of subjects who possessed more than a commonly accepted "threshold" level of each antibody, and since the number exceeding this threshold is usually 100 per cent, at least as far as the antitoxins are concerned, it is not possible to draw definitive conclusions from such reports as far as the "crowding-out" effect is concerned.

A possible useful application of this principle was suggested ten years ago by Wiener, who proposed the application of competition between antigens in an attempt to suppress the formation of anti-Rh antibodies in Rh-negative mothers (82). Various investigators, including Unger (83) and Shanaphy (84), have attempted to apply this concept. The results, however, have been disappointing—as illustrated by the fact that Wiener does not now recommend the use of this procedure (85).

One may conclude—quite tentatively since the subject is not closed—that the antibody-forming capacity of normal man is capable of assimilating a considerably larger load than it is likely to have imposed on it under any but the most extreme circumstances. This is further illustrated by the fact that diphtheria antitoxin titers in man have been observed to rise as high as 2000 units [Havens, Shaffer & Hopke (86)]. This is about 100 times as much antibody as the usual maximum range found after a booster dose and suggests, in line with innumerable other observations, that there is a wide margin of safety. Nevertheless it is to be hoped that the subject will be thoroughly explored, with methods that permit accurate evaluation of the antibody responses obtained, and with data that provide a basis for statistically sound conclusions.

#### SOME HOST FACTORS

Several factors of major potential importance in conditioning susceptibility, immunity, and the response to immunization have been covered in

previous reviews, already mentioned in this series [e.g. Gowen (2); Clark (5); Kass & Finland (8)]; this reviewer has dealt briefly with some in an earlier review (20); but there is a need for comprehensive up-to-date reviews on other aspects of the overall problem, such as the effects of radiation on immunity. Only a few aspects, brought to attention by recent published observations, will be considered here.

*Age and susceptibility.*—Sigel (7) has dealt with this factor with special emphasis on the laboratory animal. Burnet (87), in considering the significance of age as it affects infectious disease patterns in man, has brought together, largely in graphic form, an analysis of age-specific case fatality rates for meningitis, diphtheria, pertussis, measles, mumps, influenza (general), influenza (1918), typhoid fever, St. Louis encephalitis, poliomyelitis, and several special categories or geographic entities for comparison. His analyses give further emphasis to the already well-recognized minimum case fatality rates in late childhood for a number of infectious diseases, e.g. influenza, unclassified respiratory infections, measles. They also bring out unexpectedly sharp secondary peaks in young adults for influenza (1918) and typhoid fever; and, in general, they serve to point up the fact that there are markedly different patterns, with reference to age-specific case fatality, for different infectious diseases. The significance of Burnet's observations here is that they bring into focus some data from which it may prove possible to progress toward a better understanding of the relation of age to susceptibility and resistance. Likewise, an understanding of the reason behind the relatively beneficent reaction to infectious diseases of, say, pre-adolescent and adolescent children, could be of considerable value in the broader application of attenuated living agents in immunization.

The relative severity of rickettsial diseases, psittacosis, and typhoid fever (just to cite a few examples) in older persons, in contrast to the mild nature of most attacks of these diseases in children, remains unexplained. It may be that some clue to this difference will arise from further studies along the lines suggested by recent studies reported by Smith & Thomas (88) who found that young rabbits were as much as 50 times as resistant to meningococcal toxin as were older rabbits, and that the difference could be blocked by injection of colloidal iron. Should the implications of these findings be extended, showing age differences in the capacity of the reticulo-endothelial system to assimilate toxic foreign substances, an explanation for the age-specific findings referred to above might be forthcoming, and a guiding pattern for the administration of relatively toxic immunizing agents, dead or alive, might well follow.

Turning to the more familiar and frequently investigated problem of resistance in the newborn, Overman & Kilham (89) found that the high susceptibility of young hamsters to mumps infection was correlated with an undeveloped capacity to form antibodies; older hamsters, producing more antibodies, manifested only inapparent infection unless they were infected with a virus strain having a shorter incubation period, in which

case the age ceiling for apparent infection was raised. Overman (90, 91) subsequently demonstrated a close correlation between the development of the capacity to respond to mumps vaccine, and the development of resistance to frank infection with mumps virus.

*Effect of antibiotics on antibody production.*—(See also Stevens (25) for numerous references.) The observations of Smadel and his associates (26), mentioned above, provide the most quantitative of many studies in which it has been found that early and vigorous antibiotic therapy has suppressed or impaired the antibody response resulting from an infection. Such observations, however, do not provide conclusive evidence for or against the two prevailing explanations for such a finding: either the antibody response is interfered with because the production of antigen is suppressed or, possibly, antibiotics might have a direct or indirect suppressive effect on antibody production per se. Green, Wohl & Waife (92) tested the latter hypothesis by administering penicillin to 14 patients receiving TAB vaccine, running 14 simultaneous controls without antibiotic. No difference in agglutination titers could be observed either 7 or 14 days after immunization was completed. Slanetz (93) found that rats or mice injected with broth cultures of a strain of *Salmonella enteritidis* showed enhanced antibody formation following a short period of supplementation of the diet with chlortetracycline (aureomycin) or oxytetracycline. Long-term addition of these antibiotics, or chloramphenicol, to the diets, however, was associated with depression of the antibody response as compared to controls. This depression could be partially or wholly counteracted by addition of yeast or thiamin to the diet. Stevens (25), using rabbits and measuring antibody production (against radio-iodinated bovine gamma globulin) by following antigen disappearance, found that the injection of chlortetracycline, dihydrostreptomycin, or penicillin resulted in definite delay in antigen disappearance, whereas oxytetracycline was associated with a more marked delay. However, none of these antibiotics produced a change as striking as that resulting from severe restriction of protein in the diet, or x-irradiation. Also, the individual responses in the rabbits varied, all the way from no effect in some to complete suppression of antibody formation during the course of the experiment in others. Slanetz' and Stevens' interesting findings await confirmation by other investigators; if they are confirmed, it will of course be essential to determine whether the mechanism is related to a derangement of assimilation of essential nutrients or to interference with intracellular metabolism, or whether some more obscure mechanism is involved. It will then devolve upon the interested investigator to find out how far one can go in translating from rats or rabbits to man.

*Other factors.*—There is still a dearth of adequate information regarding the influence of environmental factors, serious derangements of the physiological norm, and intervening acute or chronic disease, on the capacity to form antibody or the ability to resist infection. Ipsen (94) has observed the effect of changes in environmental temperature on the antibody response of

mice to tetanus toxoid, and found that the highest level of immunity was developed in mice that were kept at the warmest temperature tested: 35 C. Havens *et al.* (95), working with human subjects, found that serious wounds, with poor healing, severe inanition, and hypoproteinemia did not result in any detectable impairment of the capacity to produce diphtheria antitoxin. These observations are in line with earlier work by Hartley, Evans & Hartley (96) in guinea pigs, and Balch (97) in man. Eichman, Miller & Havens (98) and Havens, Shaffer & Hopke (86) found, moreover, that the presence of acute or chronic hepatic disease did not interfere with the capacity of man to form diphtheria antitoxin. Their data in fact lead to the surprising conclusion that antibody formation is, if anything, enhanced in patients with liver disease.

Obviously there are many as yet unexplored, or very inadequately explored, problems similar to those illustrated by the studies just cited. Wholly aside from the protective role of such nonspecific substances as complement, and the new entity "properdin" described by Pillemer and his colleagues recently (99), both of which are known to be influenced by various pathological states, it would clarify many problems in immunization and resistance, both theoretical and practical, if the effect of such factors as acute infection, climatic stress, prolonged physical stress, and chronic or degenerative diseases on the antibody response in man could be more thoroughly elucidated.

#### RAPID DEVELOPMENT OF RESISTANCE

From time to time over the years, various investigators have noted that the inoculation of an immunizing agent resulted in the development, within 24 to 48 hours, or sometimes as early as five hours [Wright (100)], of resistance to challenge with the homologous organism. The classical findings in this respect are well summarized by Wilson & Miles (101). More recently Wharton & Creech demonstrated that resistance to the lethal effects of a toxic bacterial extract rose and declined before antibodies to the substance reached high levels (102). McCallum, Batson, Brown & Carlson (103) reported that an immune response to intraperitoneal challenge with *S. typhosa* could be detected in mice as early as 36 hours after intraperitoneal inoculation of typhoid vaccine, and that protection at two days was equal to that routinely observed at six days or later. No anti-typhoid antibodies could be detected less than six days after vaccination. Subsequently McCallum, Edsall & Carlson showed that this effect could not be reproduced if cholera or *Shigella flexneri* 3 vaccines were substituted for typhoid vaccine prior to challenge with *S. typhosa*. Typhoid vaccine did not induce any protection against *S. flexneri* 3 and only a suggestion of early protection against *Vibrio comma*, nor did the cholera or dysentery vaccines induce early protection of a significant degree against their respective homologous challenge organisms (104). During the past year Evans (105) has reported a similar type of early protection against intracerebral challenge of mice with



*Hemophilus pertussis*, detectable as early as 5 hr. after intraperitoneal injection of the vaccine and rising to a maximum at about eight to ten days, before antibodies to *H. pertussis* could be detected. Heating the vaccine to 100 C. for 15 min. destroyed its early protective effect, whereas a bacterial extract which was a poor antigen in the classical sense induced early protection comparable to that induced by an intact vaccine. In subsequent work (106), the same investigators found that intracerebral inoculation of either bacterial suspensions or cell-free extracts of *H. pertussis*, either with, or 3 hr. before, an intracerebral challenge with a strain of established virulence, afforded a significant degree of protection. They suggest that this protection may be the result of a direct cellular interference, the actual mechanism of which is not known. They consider that there may be a relationship between their observations and the protection against *H. pertussis* infection afforded by vaccinia, noted several years ago by Dalldorf *et al.* (107)—a finding which is, of course, much more difficult to explain than the observations of Evans & Perkins.

The assumption that Evans & Perkins' experiments are to be accounted for as another instance of local interference, probably based upon the presence of a specific cellular mechanism receptive to the pertussis bacillus, may have to be re-examined in the light of Rowley's recent report on the effects of bacterial cell walls and zymosan in altering the relative virulence of *Escherichia* for mice (108). His findings, which appear to be directly related to the presence and properties of properdin (99), disclosed a marked drop in resistance of mice immediately after an injection of the cell-wall preparations or zymosan, followed by a "rebound" effect so that in 24 hr. the mice had become exceptionally resistant to the same challenge organism. However, the possible relation of these findings to the experimental observations of Evans & Perkins (105, 106) and those of McCallum *et al.* (103, 104) will have to await further quantitative and chronologically precise titrations of the zymosan (or properdin?) effect, in the various experimental patterns concerned, before a choice between hypotheses can be made.

A number of experiments, in which tetanus and possibly diphtheria toxoids have shown an analogous protective effect, have led to a different concept of "early immunity." Cremer (109) and Grote (110) interpreted some earlier experimental studies by Schmidt & Scholz (111) as indicating a direct tissue-receptor competition between diphtheria toxin and toxoid. They translated this concept into the treatment of clinical diphtheria with toxoid injections; however, their results, which lack controls, do not provide evidence that the toxoid had any effect, either in blocking diphtheria toxin from attachment to susceptible cells, or in releasing it following its fixation. Then Krech reported that tetanus toxoid, injected 24 or 48 hr. before challenge of mice with tetanus spores, resulted in protection of the mice (112). He concluded that the toxoid induced rapid formation of antibodies.

Lemétayer and his colleagues demonstrated that definite delay in the symptoms of tetanus ensued if toxoid was mixed with toxin prior to injection.



tion (113) and that clear-cut delay in symptoms resulted even if the toxoid was separately injected 5 hr. after the toxin (114). In further quantitative studies Raynaud, Lemétayer *et al.* showed that three daily doses of toxoid, 200 Lf each, begun one day before the toxin was injected, fully protected mice against one lethal dose of toxin (115). To overcome the obvious objections to these experiments, Raynaud & Wright (116) repeated them, using toxin and toxoid from different laboratories, injecting the toxoid intravenously, and including diphtheria toxoid as a control. Toxin was injected from 1 through 6 days after toxoid. It was found that 200 Lf of tetanus toxoid protected against 1 but not 4 m.l.d. of toxin—a toxoid/toxin ratio of about  $4 \times 10^6$  therefore being required to be effective. The protection fell off on the second day and was gone by the sixth day after toxoid injection, thus excluding the possibility that it was due to conventional antibody formation. Diphtheria toxoid gave no protection, even under conditions found optimal for tetanus toxoid. These authors quite logically suggest that the mechanism of action of the toxoid is either a blocking of a hypothetical receptor substance in the susceptible host cells, or a competitive inhibition of toxin by toxoid. Goldman *et al.* (117) have essentially confirmed the findings of Raynaud & Wright without, however, specifying the potency of the toxoid they employed.

It might be suggested that at least some of the various early protective effects described above were analogous, in principle, to the non-species-specific tolerance of bacterial endotoxins demonstrated in animals some years ago by Zahl & Hutner (118) and in man by Morgan (119). However, the apparent specificity of the effects of tetanus toxoid on the one hand and typhoid vaccine on the other, and the efficacy of locally (i.e. intracerebrally) administered pertussis vaccine, would argue against such a postulate. It would appear, however, that each experimental pattern requires thorough definition as regards timing, specificity, and other factors; and that it may well turn out that there are several mechanisms involved in the rapid development of resistance. Furthermore, the well-established fact that antibody formation may occur in small animals such as mice and rabbits as early as two or three days after injection of an antigen<sup>6</sup> must be borne in mind in planning or interpreting experiments pointed to the elucidation of the phenomenon of "early immunity."

<sup>6</sup> For classical observations on this point see Wilson & Miles (120); for an example of recent findings, see Larson *et al.* (121).

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# THE STRUCTURE OF ANTIGEN-ANTIBODY AGGREGATES AND COMPLEMENT FIXATION<sup>1</sup>

By JOHN MARRACK

*Department of Pathology, University of Cambridge, Cambridge, England*

## STRUCTURE OF ANTIGEN-ANTIBODY AGGREGATES

The aggregates formed by antibodies and antigens have two remarkable properties—first, they “fix” complement and, second, the “fixation” of complement by aggregates of antibody and antigen on the surface of red blood cells is followed by lysis of these cells. These properties have been exploited in diagnosis for over 50 years but are still little understood; this is not surprising as we have still much to learn about the fundamental structure of aggregates of antigen and antibody. Before going on to new and interesting developments of the study of the secondary and tertiary processes, we may try to get some idea of what these aggregates are like.

*Structure of proteins.*—Antibodies are proteins and the antigens mainly used in the study of the aggregation have been proteins. Largely through the work of Pauling and his school our concepts of the possible basic structures of proteins are much more precise and restricted than they were when he wrote about the formation of antibodies in 1940 (1). The number of possible configurations of peptide chains is limited (2); of those considered, the  $\alpha$ -helix of Pauling & Corey (3) seems open to fewest objections. Essentially the molecules of “globular” proteins are bundles of parallel rods about 10 Å in diameter. The rods have a skeleton consisting of a peptide chain,  $-\text{CO}-\text{NH}-\text{CH}-\text{CO}-\text{NH}-\text{CH}-$ , arranged in this helix and surrounded by the tails of the constituent amino acid residues. Sanger's method (4) of estimating the number of N-terminal amino acids shows that several independent peptide chains may be included in one molecule. The molecules have a more complicated structure than that of the model of the haemoglobin molecule first proposed by Perutz (5); and, where the chains are folded on themselves, the peptide chains may take the ribbon configuration advocated by Robinson & Ambrose (6). As there are about 3.7 amino acids per turn of the helix, the tails of the amino acid residues may assume a great variety of orientations with respect to the surface of the molecule. The properties of the protein must depend on the nature, sequence, and orientation of these tails.

There is no reason to suppose that the molecules of proteins are ellipsoidal. The dimensions calculated on this assumption are those of an “equivalent ellipsoid of revaluation” chosen to fit as closely as possible the actual behavior of a molecule in solution [Edsall (7)]. The dimensions of the “equivalent ellipsoid” may be fairly close to true dimensions. Thus, Low (8) concluded from x-ray studies of crystals that the molecule of human serum

<sup>1</sup> The survey of literature pertaining to this review was completed in November, 1954.

albumin is a prism approximately 145 Å long, nearly 50 Å in diameter at the center, and 22 Å thick; the axes of the equivalent ellipsoid are 160 Å and 37.5 Å (9). Molecules of human serum  $\gamma$ -globulin are longer; the axes of the equivalent ellipsoid are 235 Å and 47 Å. If, as is probable, the molecule is irregular and does not taper at the ends, it is not so long. The dimensions of rabbit  $\gamma$ -globulin (mol. wt. 160,000) are very similar (10).

*Combination of antigen and antibody.*—The combination of artificial antigens, such as azoproteins, with the corresponding antibodies involves the close approach of a pattern of antigen atoms to a complementary pattern of antibody atoms. Pauling & Pressman (11) consider that the determinants of an antigen fit into crypts on the surface of the antibody molecule. This combination of determinant with antibody may be similar to the adsorption of organic anions, such as methyl orange, by serum albumin; Karush (12) has recently suggested that the anions fit into gaps between parallel helices of the albumin molecule. We have every reason to suppose that the combination of natural protein antigens with the corresponding antibodies also involves a close approach of complementary patterns of atoms. For such a multipoint attachment it is essential that the antibody molecule should be correctly oriented with respect to the antigen molecule; no rotation round the bond is possible.

There is no evidence to support the claim [e.g., Haurowitz (13)] that the nature and sequence of the constituent amino acids of antibodies do not differ from those of "normal"  $\gamma$ -globulin. Gamma-globulins are a family of proteins that differ in electrophoretic mobility; those of bovine and human serum include molecules with different N-terminal amino acids (14). There is no "normal"  $\gamma$ -globulin with which antibodies can be compared. Calvery (15) found a significant difference between the amounts of cystine in antibodies against SI and SII. Since the number of configurations of peptide chains is limited, we can rule out Pauling's original proposition (1) that the specific combining sites of antibodies can be formed by appropriate coiling of the ends of a peptide chain after synthesis. Porter (16) found that the terminal tetrapeptide of rabbit anti-ovalbumin did not differ from that of inert  $\gamma$ -globulin; Orlans (17) also found the same N-terminal amino acid in rabbit anti-bovine-serum-albumin and inert  $\gamma$ -globulin and inferred that the end of the peptide chain could not form part of the combining groups of the antibody. It is, however, possible that the combining group may lie at the end of the molecule.

All models of protein molecules so far proposed [haemoglobin (5, 18), insulin (19), serum albumin (1)] have distinct dissimilar surfaces; the specific structures that form the combining groups of protein antigens cannot be distributed promiscuously over the surface of the molecules, as has been assumed in recent discussions (20).

*Long-range forces.*—We may turn aside here to mention Rothen's contention (21) that long-range forces are involved in the interaction between anti-



bodies and antigens and that these forces may act through layers of inert substances. This claim has been criticized by several writers (22 to 25) on the grounds that the films were uneven, that there may be holes in the layers of inert substance used, and that antigen or antibody may be leached out from the films. McGavin & Iball (26) consider that some factor in protein solutions promotes re-resolution of antigen films. There seems to be no reason to suppose that antigen and antibody are deposited in neat layers one on the other, rather than that antigen and antibody aggregate as usual and that the combined aggregates form a film.

*Valence of antibodies.*—Boyd (27) pointed out in 1947 that only one study (28) had given direct evidence that an antibody molecule could combine with two molecules of antigen. Since then, several investigations (29 to 32, 34) have shown that in antigen excess one antibody molecule can combine with two molecules of antigen or haptene. Becker (33) has deduced the molecular weight of the antigen-antibody compound formed in antigen excess, from sedimentation and diffusion constants, and found the sedimentation constants of the compound formed with horse antibodies higher than those of compounds formed with rabbit antibodies; this might be due to greater asymmetry of the horse-antibody-antigen compounds. Singer & Campbell (30) also found evidence of asymmetry of the compound containing one molecule of antibody and two of antigen, such as would be due to location of the combining sites at the ends of the antibody molecule. We may therefore assume that antibodies are bivalent; it follows from the composition of antigen-antibody precipitates, formed in antibody excess, that antigens are multivalent.

*The structure of antigen-antibody aggregates.*—Once this question of the valence of antibodies was settled it followed that, when the ratio of antibody to antigen was suitable, aggregates would be formed consisting of antibody molecules linked to two antigen molecules and antigen molecules linked to several antibody molecules. There are two concepts of the structure—on the one hand, branched chains of alternating antigen and antibody molecules; on the other, cyclic structures (hence the name lattice) envisaged by Heidelberger & Kendall (35) and Marrack (36). It would be as well if the term "lattice theory" were reserved for those theories that propose that a lattice is actually formed and to use the term "alternation theory," proposed by Hooker & Boyd (37), for the chain theory.

Many theories of the formation of antigen-antibody precipitates seem open to the objection that they assume that bonds once formed are irreversible. This difficulty is overcome in Goldberg's treatment (39). He applies the statistical methods of calculating the composition of branched chain polymers introduced by Flory (40) and further developed by Stockmayer (41); see also (42). The theory assumes that the aggregates formed will be those that can be made in the largest number of ways and have therefore a high probability of formation or entropy. All reactions are reversible; the

theory does not require bonds to be fixed once they are formed. The aggregates so formed are mainly branched chains, with very little formation of cyclic structures.

This treatment leads to the conclusion that aggregates of antigen and antibody will continue to grow up to a critical point, at which a few large aggregates are formed by the union of numerous aggregates of moderate size; these large aggregates precipitate. This critical point will be reached only when the ratio of total antibody to total antigen lies within certain limits; precipitation, due to the formation of these large aggregates, should occur only when the ratio lies within these limits. Hence, there should be a prozone as found with horse antisera reacting with protein antigens (43 to 47), though not with all protein antigens<sup>2</sup> (48, 57) or with polysaccharides. The best agreement between calculated and experimental results is that with diphtheria toxin and horse antitoxin found by Pappenheimer & Robinson (43). Pappenheimer & Robinson considered that their curve was given by a single antigen, with a ratio of  $\mu\text{g. N per Lf}$  very close to that of the purified toxins of Eaton (48) and Pappenheimer (49). However, several workers (50 to 53b) have shown that purified toxins contain several antigens and that antitoxic sera contain several antibodies. And Pope & Stevens (54) and Pope (55) have produced evidence that the Pappenheimer & Robinson type of curve is a composite curve made up of the curves of several antigen-antibody systems. So that no conclusions can be based on the agreement of Goldberg's calculations with the results of Pappenheimer & Robinson.

However, the horse type of precipitation curve is exceptional; sera of other species, including donkeys (56), give "rabbit" type curves, forming precipitates even in extreme antibody excess; and these are the sera with which we are mainly concerned. Goldberg (39) attributes this precipitation outside the critical limits to the lower solubility of rabbit antibody, quoting Boyd (59). Pope's demonstration (60) that, in 20 per cent ammonium sulphate, diphtheria toxin and horse antitoxin give a "rabbit" type curve supports this contention. But Boyd refers to solubility in concentrated ammonium sulphate; solubilities in 0.15 *M* NaCl do not necessarily differ in the same way. Molecules of antibody must be closely packed in the compounds formed in antibody excess. The molecular ratio of antibody to antigen in the precipitate formed by rabbit antibody with bovine serum albumin in great antibody excess is 7 (38). Even if all the antibody molecules are combined with one antigen molecule only, both the largest surfaces (length about 145 Å and greatest width 50 Å) (8) of the antigen molecule must accommodate at least three antibody molecules with diameters of about 45 Å. Polar groups of the molecules may be occluded with consequent loss of solubility.

Although when smaller molecules form polymers, the formation of cyclic structures is excluded, we may contemplate their formation when the large molecules of antigen and antibody aggregate. Pope (60) has observed that

<sup>2</sup> The type of curve does not depend on the route by which horses are immunized (58).

when antigen and antibody first combine they form a gel extending throughout the solution. Some rearrangement must take place in the subsequent formation of relatively compact precipitates. If we are right in believing that no rotation can occur round the bond between an antigen and an antibody, this rearrangement can only be produced by dissociation and recombination, possibly to form more stable cyclic structures. The location of combining sites on the molecules and their relative orientations may be such that cyclic structures are inevitable. The difference between "horse" and "rabbit" types of precipitation curves may be due to differences in the relative orientations of combined antigens and antibodies as suggested by Becker (33).

Some evidence about the structure of antigen-antibody aggregates can be got from study of the precipitates. After prolonged centrifuging the precipitates contain some 86 per cent of water; of this some 80 per cent is available for solution of haemoglobin (61), so that there is room for such large molecules in all but some 20 per cent of the total water. Smaller molecules must be able to penetrate the aggregates rapidly, for various substrates—urea (62), tyrosine (63), and  $H_2O_2$  (64)—are broken down by enzyme-antibody precipitates nearly as fast as by the free enzymes. What is more surprising is that the rate of breakdown of the large molecules of ribonucleic acid by ribonuclease-anti-ribonuclease precipitates is nearly as fast as the rate of breakdown by the free enzyme (65); but in this case the ratio of antibody to antigen molecules is not more than 3 to 1.

We may, therefore, consider the aggregates to be composed of constellations of antibody molecules fairly closely packed round an antigen molecule. Some of the antibody molecules serve as bridges between two constellations linking them into branching chains and possibly loose networks, but not into a tightly packed lattice.

*Incomplete antibodies.*—Goldberg (39) considers the possibility that some antibody molecules may be univalent and calculates the effect of varying ratios of univalent to bivalent antibody on prozones and postzones. His conclusions agree well with the effects on agglutination of varying ratios of incomplete to complete anti-Rh antibodies found by Sturgeon (66). However, Heidelberger, who introduced the term "univalent," states (46) that it is not used in the literal sense, and Kabat (67) has criticized the evidence for univalence that has been produced. As Marrack & Grant (68) point out, complete antibodies may become incomplete in low salt concentrations and any factors that oppose aggregation may inhibit it before large aggregates are formed. Until we have some satisfactory evidence of the existence of univalent antibodies, it would be advisable to stick to the term incomplete, that carries no implications.

#### COMPLEMENT

According to Browning (69), Muir (70) defined complement as "that labile substance of normal serum which is taken up by the combination of an antigen and its anti-substance (immune body)." This definition, as Brown-

ing wrote, is of wide application. There is good reason for using the term in a wide sense. For complement may be "fixed" (that is, made unavailable for the lysis of other red-blood-cell-antibody systems) by sensitized red blood cells, which, however, are not lysed. For example, horse complement, that lyses rabbit red blood cells sensitized with sheep antibody (130) and ox cells sensitized with cat antibody, is fixed by ox-red blood cells sensitized with rabbit antibody, although the fixation is not followed by lysis (71). Something analogous to complement is involved in the conglutination reaction of Bordet & Streng (114), so that lysis of cells is not essential. Also, components similar to those of haemolytic complement take part in reactions in which no antigen-antibody is involved—the inactivation (92, 93) of one of the complement components (C'3) and the action of plasmin (95 to 97). Again antigen-antibody precipitates take up a certain amount of protein from active serum but not from serum that has been inactivated by heat (72). The question arises, are the same constituents involved in all these reactions? For example, is the horse C'1 involved in lysis of rabbit red blood corpuscles sensitized with sheep antibody (125) and "fixed" by ox-red cells sensitized with rabbit antibody, the same as the C'1 involved in conglutination (121), and is it the same as the protein that an ovalbumin-rabbit-antibody precipitate takes up from fresh horse serum (74)? Is this uptake of protein chemical evidence of the "fixation" of complement?

Let us start with this "fixation." For over 50 years it has been known that complement is inactivated by antigen-antibody complexes. The activities of C'1, C'2 and C'4 are reduced or abolished, while C'3 is less affected (75 to 77, 97). Inactivation does not necessarily mean that the components are actually combined with the antigen-antibody complex. The changes that lead to the eventual lysis of red blood cells may be produced without permanent combination of complement components with the cells. The possibility that C'1, C'2 and C'4 are inactivated rather than combined is suggested by the work on the action of plasmin (95 to 97) discussed later. The only experiment, that shows that one of the haemolytic components is actually taken up, is one made by Dean (78) over 40 years ago and, as far as I know, never repeated. He showed that haemolytically active mid-piece could be recovered from the washed precipitate that had been formed by horse serum (as antigen) with rabbit antiserum in the presence of fresh guinea pig serum.

*Protein uptake.*—Heidelberger's original experiments (72) showed an unquestionable difference between the amounts of precipitates formed in the presence of active and of heat-inactivated guinea pig serum. Thus, in his first experiment, the amounts of N in the precipitates formed by SIII with rabbit antiserum in the presence of 5 ml. of saline, of heat-inactivated guinea pig serum, and of active guinea pig serum were 588, 600, and 720  $\mu$ g. The later experiments of Maurer & Talmage (80) with I<sup>131</sup>-labelled antigen and antibody have shown that this increase in the amount of protein precipitated is not due to more complete precipitation of antigen and antibody,

except in the region of antigen excess.<sup>3</sup> This uptake of protein by an antigen-antibody complex may, at first sight, not seem remarkable. But one of the striking features of washed antigen-antibody precipitates is that they are remarkably free from contamination with nonspecific soluble proteins present in the solutions in which the precipitates formed. This has been shown repeatedly with proteins that can be recognized, such as haemoglobin (82), azoproteins (83) and proteins labelled with  $I^{131}$  (84). Apart from the great difference between the amounts of protein taken up from active serum and from heat-inactivated serum, the strongest evidence that the protein taken up is complement is the failure of the precipitate formed by pneumococcal polysaccharides with horse antisera to take up protein from active guinea-pig serum (72); these horse antibody-polysaccharide precipitates also do not "fix" complement. The precipitates formed by rabbit antisera with these polysaccharides fix complement and take up as much protein from active guinea pig serum as is taken up by ovalbumin-antibody precipitates (72).

$Ca^{++}$  ions have a varying effect both on "fixation" of complement and on protein uptake. Lepow and colleagues (97) found that the effect of removal of  $Ca^{++}$  ions by a cation-exchange resin on the "fixation" of complement components of human sera varied both with the amount of antigen-antibody complex used (when large amounts of complex were added, removal of  $Ca^{++}$  had little effect) and with the serum; they considered that  $Ca^{++}$  ions were not an absolute requirement. On the other hand, Levine and colleagues (105) reported that complements of guinea pig serum and one human serum from which  $Ca^{++}$  ions had been removed by a chelating agent were not fixed complement. Maurer & Weigle (81) found that, if the  $Ca^{++}$  ions were removed from complement-containing sera by a chelating agent, antigen-antibody complexes did not take up protein from guinea pig serum or from one human serum, but took up a reduced amount from a second human serum and the same amount from a rabbit serum as before the  $Ca^{++}$  ions were removed.

The protein taken up by antigen-antibody precipitates is mainly derived from mid-piece (72, 85); if it is a component of complement, it must be mainly C'1. Now the amounts of protein that are taken up per ml. from guinea pig (72, 73), human (73, 74), bovine (86), pig (87), horse (74) and rabbit serum (79) are of the same order; the amounts of C'1, active in lysis of sheep red cells sensitized with rabbit antibody, were found by Rice & Crowson (88) to be, in haemolytic units per ml.: guinea pig 3,750; human, 4,000; bovine, 500; pig, 1,720; horse, 500. Also antigen-antibody precipitates take up much the same amount of protein from rabbit sera that have been kept for months at 0° and have lost all haemolytic activity, as from fresh rabbit serum (79, 80). There are three possibilities: first, that the sera of these species all contain about the same amount of special proteins that have the

<sup>3</sup> Maurer & Talmage (80) found that less antibody might be precipitated by antigen when the mixture was diluted with fresh or decomplexed serum than when diluted with saline. This may account for some of the conflicting results and the puzzling effects of heated serum found by Johns (74).

property of being taken up by antigen-antibody precipitates and vary in their haemolytic activity, just as the toxicity of diphtheria toxin is independent of its ability to combine with antitoxin; secondly, that the actual C'1 forms a part, possibly small, of the protein that is taken up by the precipitates; and, thirdly, that the uptake of protein is independent of the "fixation" of complement which is merely inactivated.

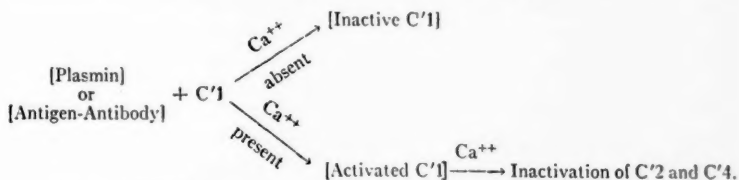
*The inactivation of C'3.*—The inactivation of C'3 by yeast and cobra venom is an example of a reaction in which components analogous to those of complement partake, although antigen-antibody reactions are not involved. Nathan (89) and Misawa (90) had shown that neither cobra venom nor yeast inactivate the C'3 of heated serum, and Da Costa Cruz & Avenedo Penna (91) had found that addition of a small amount of fresh guinea pig serum to heated serum restored the ability of yeast to inactivate C'3. Pillemer and colleagues (93), using the yeast polysaccharide zymosan and human serum, found that this ability was restored by any mixtures of the reagents R1, R2, and R4 (see 93a) that would effect haemolysis and that R3 (that is, the reagent that contains C'1, C'2 and C'4) was effective by itself. Unlike Mutsaers & Barthels-Viroux (94), who used cobra venom as inactivator, Pillemer and colleagues (93) considered that the necessary constituents of serum included C'4, as C'3 was not inactivated by zymosan when C'4 had been inactivated by hydrazine; they concluded that C'4 was not as fully inactivated by ammonia (which Mutsaers & Barthels-Viroux used) as by hydrazine. Mutsaers & Barthels-Viroux found that serum from which all haemolytic activity had been removed by absorbing with sensitized red blood cells or stromata was still effective in restoring inactivation of C'3 by cobra venom; but, according to Pillemer *et al.* (93), the inactivating substances are removed by rabbit-antibody-SIII precipitate. It is possible that the components were not completely removed by the sensitized red cells and that more of these components was needed for haemolysis than for inactivation of C'3.

The substances in fresh serum, involved in this inactivation of C'3, therefore resemble three components of complement. Another similarity of this inactivation of C'3 to complement action is its dependence on  $Mg^{++}$  ions (92), like one stage of lysis of sensitized red blood cells by complement (107). As  $Mg^{++}$  ions are essential for the inactivation by zymosan and as the rate of inactivation rises rapidly as the temperature is raised from 0° to 37°, Pillemer and colleagues (92) consider that the inactivation resembles an enzyme action rather than an adsorption.

*Action of plasmin on components of complement.*—Plasmin, the proteolytic enzyme of serum, which in human serum is activated at 37° by streptokinase, rapidly inactivates the C'2 and C'4 of untreated human serum (95) and has less effect on the C'1 and little on the C'3. In R'1, the reagent that contains no C'1, C'2 is little affected by plasmin and C'4 is inactivated much less than in serum; but the C'2 is inactivated in R'3 and R'4 and the C'4 in R'2 and R'3. C'1 or some constituent associated with it is therefore necessary

for the inactivation of C'2 and C'4 by plasmin. In serum, that has been freed from  $\text{Ca}^{++}$  ions by passage through a cation exchange resin, C'1 is inactivated on incubation at  $37^\circ$  even without plasmin and more completely if plasmin is present; on the other hand, in the  $\text{Ca}^{++}$  free serum, C'2 and C'4 remain fully active without plasmin and are little inactivated with plasmin (96). With increasing amounts of  $\text{Ca}^{++}$  added to resin treated serum, the C'1 is progressively less inactivated on incubation and C'2 and C'4 progressively more inactivated by plasmin.  $\text{Ca}^{++}$  ions, therefore, have a protective action on C'1 and the effect of removal of  $\text{Ca}^{++}$  on the inactivation of C'2 and C'4 by plasmin is indirect. The addition of very little C'1 is needed to bring about the inactivation of C'2 and C'4 in R'1 by plasmin; this may be compared with the very small requirement of added fresh serum in inactivation of C'3, in heated serum, by venom or zymosan.

On the strength of the dependence of inactivation of C'2 and C'4 by plasmin on the presence of C'1 and  $\text{Ca}^{++}$  ions, Pillemer and colleagues (97) suggest an interrelationship between the plasmin-complement and antigen-antibody complement systems represented by the scheme:



C'1 is assumed to be a proenzyme that can be converted to an active enzyme by plasmin and by antigen-antibody aggregates. Complement "fixation" would therefore be an enzymic reaction in which an antigen-antibody aggregate would serve as a catalyst for the conversion of proenzyme to active enzyme.  $\text{Ca}^{++}$  would be required to prevent spontaneous inactivation of C'1 and also, perhaps, to enhance the activity of "activated C'1" (97). It would be necessary to postulate the eventual complete inactivation of C'1, otherwise all the C'2 and C'3 would eventually be destroyed.

There are, however, serious differences between the inactivation of complement by plasmin and complement "fixation" by antigen-antibody aggregates. In the first place, C', including C'1, is fixed by antigen-antibody aggregates at  $0^\circ$ , whereas inactivation by plasmin takes place only at higher temperatures. In the second place, there is no indication in the experiments of Levine and colleagues (107) that C'1 is inactivated when  $\text{Ca}^{++}$  free serum is incubated, but only that its action is temporarily suspended; therefore, the failure of complement "fixation" in the absence of  $\text{Ca}^{++}$ , in those instances in which "fixation" does fail, is not due to inactivation of C'1.

Pillemer and colleagues (97) cite the alleged activation of plasminogen by antigen-antibody aggregates as a process possibly comparable to some stage of complement "fixation." However, plasminogen is not activated by

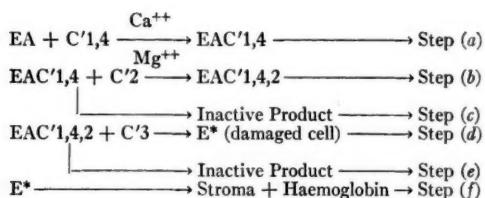


aggregates of ovalbumin and antibody (98). The activation of plasminogen by antigen-antibody aggregates, described by Ungar and colleagues (99) in their latest paper, seems very different from complement "fixation." For they found that the amount of antigen (egg albumin) optimum for activation of plasminogen was over 1 mg. (up to 5 mg.) per ml. of the serum of a guinea pig that had received a single injection of antigen subcutaneously. One mg. of antigen would be more than the equivalent amount if the antiserum contained as much as 5 mg. of antibody per ml; actually the antisera examined contained well under 1 mg. of antibody per ml. Thus the amounts of antigen optimum for activation of plasminogen were far in the region of antigen excess, whereas optimum amounts of antigen for complement fixation are about equivalent to the antibody in the serum (100).

### THREE STAGES OF HAEMOLYSIS

Seven years ago Mayer and colleagues (101) suggested that the combination between haemolysin and red blood cells is reversible and that haemolysin molecules can move on from a cell that has been lysed to combine with another cell and promote its lysis if sufficient complement is available. This migration of haemolysin would explain the observed fact that, if enough complement is present, lysis is a progressive process although the amount of haemolysin is limited. Bowman, Mayer & Rapp (102), using a method rather similar to those of Philosophow (103) and Muir (104), showed that the haemolysins could be released from sensitized stromata and lyse added red blood cells.

The most recent work of Mayer, Levine and colleagues (105 to 109c) has divided the process of haemolysis into clear-cut stages and solved some of the problems connected with the estimation of the components. They recognize four steps after the combination of red blood cells (E) with antibody (A) to form EA:



The first step takes place at 0° in the presence of  $\text{Ca}^{++}$ ; the second step also takes place at 0° but only if  $\text{Mg}^{++}$  is added. So that both EAC' 1,4 (109a) and EAC' 1,4,2 (109b) can be prepared, using serum from which  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  have been removed by passage through a cation-exchange resin and adding the amounts of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  required for the two stages. Step c has a high temperature gradient and is very slow at 16°; so that EAC' 1,4,2 does not go through the next stage at 0°. The first step is relatively

slow, reaching maximum formation of EAC' 1,4 in about 6 hr. at 0° and about 8 min. at 37°; but the number of cells lysed on subsequent incubation with C'2 and C'3 is less after incubation at 37° owing, apparently, to inactivation of EAC'1,4 at this temperature (109a). EAC'1,4,2 is more unstable, having a half life at 37° of about 10 min. The rate of formation of EAC'1,4,2 from EAC'1,4 depends on the concentration of C'2, and the degree of haemolysis depends on the C'3 available to deal with the EAC'1,4,2 formed before it is inactivated (109c). The apparent titer of C' and of the components depends on the relative amounts of the other components. A reagent C'y, containing C'2 and C'3, could be prepared (109a) by fixing the C'1 and C'4 of a guinea-pig serum with a serum-albumin-antibody precipitate at 0° in absence of  $Mg^{++}$  (C'2 is not fixed in the absence of  $Mg^{++}$  by this precipitate formed by protein antigen and antibody). From the relation of the rate of lysis of EAC'1,4,2 to the concentration of C'3 the authors concluded that 2 molecules of C'3 react simultaneously with EAC'1,4,2 to yield E\*. By use of C'y they showed that some protein of C'y, presumably C'3, combined with EAC'1,4,2 at 0° (109c).

A plausible theory of this terminal stage would be that the antibody has combined with some antigen that is a constituent of the cell surface, and that complement induces some further change in this antigen-antibody compound that affects this structure of the cell surface. However, it is not necessary that the antigen should be a normal constituent of the cell surface, so long as it is attached to the cell. Thus Silverstein & Maltaner (111) coupled diazotized *p*-aminophenylarsonic acid to sheep red blood cells and found that these cells were lysed by an antiserum against the proteins of beef serum coupled with *p*-aminophenylarsonic acid together with complement. The reaction was specific. No lysis was found with the antiserum against a *p*-aminobenzoic acid antigen. Further, Fisher & Keogh (112) and Adler (113) have shown that red blood cells that have adsorbed various antigens fix complement and are lysed in the presence of antisera against the adsorbed antigens. The antigen, therefore, need only be attached to the red cell; the lysis resembles a cellular anaphylaxis.

#### CONGLUTINATION

Conglutination was the name given by Bordet & Streng (114) to describe the agglutination of red blood cells, that had been sensitized by an antibody and had absorbed complement, by a heat stable substance, which they called conglutinin, present in the serum of normal bovines.<sup>4</sup> A heat labile substance, analogous to haemolytic complement, is essential; this may be called conglutinating complement. The use of the word "conglutination" as a name for wholly different reactions that do not involve "complement" leads to need-

<sup>4</sup> A similar phenomenon was described by Muir & Browning (118). They used ox red blood cells sensitized with rabbit antibody; ox serum supplied both complement and conglutinin.

less confusion. An admirable review of the earlier investigations on the subject has been published by Hole & Coombs (115).

As the reaction was first described by Bordet & Gay (117), bovine serum (heated at 56° for one-half hour) and fresh horse serum were added to a suspension of sheep red blood cells; the red cells were then agglutinated in large clumps. The bovine serum supplied two factors, the natural antibody for sheep red cells present in bovine serum and the conglutinin; the horse serum supplied the complement. Other particulate antigens can be used and conglutination has been exploited in diagnostic tests, both as a direct reaction and as a complement fixation reaction (116).

Streng (119) and Wartiovara (120) have obtained antisera that react like conglutinin by immunizing rabbits with particles that have "fixed" complement; they call the active substance in such antisera immunoconglutinin. This immunoconglutinin is non-specific. Like conglutinin it will promote agglutination of particles that have "fixed" the conglutinating complement of several species. Its action is not limited to particles that have "fixed" the conglutinating complement of the species to which the complement on the immunizing particles was obtained. Immunoconglutinin is not only formed in response to injections of sensitized particles that have fixed complement *in vitro*, but also in response to intravenous injection of bacteria free from antibody and complement when injected (120); it is supposed that the bacteria take up both antibody and complement from the plasma of the animal into which they are injected. By absorption tests Coombs & Coombs (121) distinguished between immunoconglutinin, on the one hand, and, on the other hand, antibody against free serum proteins (of the serum from which the complement was derived) and against the particles on which the complement was carried. They suggest that the proteins of the complement undergo some physical change when fixed and that, with this change, the proteins acquire a new specificity foreign to the species from which the proteins were derived.

In further discussion of conglutination it will be assumed that conglutinin is an antibody against some antigen, derived from serum that supplies the analogue of haemolytic complement and modified after attachment to the antigen-antibody aggregate. This is not, necessarily, evidence that complement itself is actually fixed by the antigen-antibody aggregate rather than being inactivated; for we know, from the uptake of protein by antigen-antibody complexes, that some antigen, not necessarily a constituent of complement, does become attached to antigen-antibody aggregates. However, Rice (122) showed that two components analogous to haemolytic C'1 and C'2 are required for conglutination; Coombs and colleagues (123) showed that C'4 also was involved. So to avoid piling up cumbrous hypotheses we may suppose that at least C'1 is actually attached to the antigen-antibody aggregate.

Blomfield (124) not only showed that a C'4 (that is, a component inactivated by ammonia) was involved but also that, of the sera of the different

species (bovine, horse, cat, pig, man, guinea pig, and rabbit) tested, only bovine and horse serum contained a C'4 that would conduce to conglutination and that the C'4 activity of bovine serum was much the highest. She also found that guinea pig C'4 has an opposing action, for sensitized red blood cells to which ammonia-treated guinea pig serum had been added were conglutinated by heated bovine serum by virtue of the bovine C'4, whereas, if untreated guinea pig serum were used, the cells were not conglutinated in spite of the presence of the bovine C'4. The C'1 in the sera of most of the species tested by Rice & Crowson (88) was active in lysing sheep red blood cells (sensitized with rabbit antibody) in conjunction with guinea pig C'4. So that it appears that the C'4 of horse and bovine serum are active in conglutination but not in lysis of this particular red-cell-antibody system whereas C'4 of guinea pig is active in lysis of this particular system but opposes conglutination. Horse and bovine serum lyse rabbit red blood cells sensitized with sheep antibody and C'4 active with this system can be demonstrated in horse and bovine serum (125). Therefore, C'4 components cannot be divided into lytic and conglutinating C'4s; rather, C'4s may be active with some systems and not with others. The relative titers of sera of different species and of the components of these sera differ according to the system used in their measurement (88, 122, 125). Blomfield (124) found that the supernatant fluid from a mixture of serum (horse, cat, pig, and man) with an equal volume of 2.8 M ammonium sulphate was inactive in conglutination of red cells sensitized with bovine antibody and active in conglutination of cells sensitized with rabbit antibody; she considered this to be evidence of two different C'1 components, one precipitable by 1.4 M ammonium sulphate and the other not. However, Blomfield, unlike Pillemer and colleagues (126) added the ammonium sulphate directly to the serum, instead of diffusing it in from a dialyzing sac in the cold. Browning & Mackie (127) and Parsons (128) have shown that this is an unsatisfactory way of separating C'1 from C'2 and it is possible that enough C'1 was left in the supernatant to be effective with one antibody but not with the other.

We see that estimates of the activities of the components of complement vary with the indicator system used and the conditions of estimation. Unless we are to multiply entities indefinitely we must suppose that units per ml.—estimated by Hegedüs & Greiner (129), Bier *et al.* (93a), Jonsen *et al.* (87), Rice & Crowson (88), and Rice (122)—are measures of particular activities and not of actual amounts of certain substances.

We have, then, a loose network of antibody and antigen molecules. We may consider that the basic reaction with a complement-containing serum is fixation of certain protein molecules, including C'1 and some C'4, in the network. These molecules are not merely trapped, as they can enter preformed antigen-antibody precipitates. The apparent failure of antigen-antibody aggregates to fix complement after they have reached a certain size is due to slowness of penetration (74). Heidelberger & Mayer (76) have suggested that complement molecules have a weak affinity for  $\gamma$ -globulin and cite the

anticomplementary effect of  $\gamma$ -globulin (130), although we do not know whether  $\gamma$ -globulin inhibits the fixation of complement rather than the subsequent lysis of red blood cells. We may accept the second half of their suggestion and suppose that molecules of C'1 and some C'4 fit into gaps between antibody molecules and are held by nonspecific attractions between atoms brought close together. Some antigen-antibody aggregates fail to fix complement because the shapes and orientations of antibody molecules are such that the gaps between them are not of suitable shape. If C'4 is not present when C'1 is fixed, the complement will not effect lysis of red cells (131). Hence we may suppose that C'4 induces some distortion of adjacent molecules with the result that C'1 is activated, as postulated by Lepow *et al.* (97), and acquires a new immunological specificity, as suggested by Coombs & Coombs (121). This would be the  $\text{Ca}^{++}$  step of Mayer & Levine (108).

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## THE RESISTANCE OF BACTERIAL SPORES WITH REFERENCE TO SPORE GERMINATION AND ITS INHIBITION<sup>1</sup>

By C. F. SCHMIDT

*Metal Division Research & Development Department, Continental Can Co., Inc.,  
Chicago, Illinois*

The resistance of bacterial spores has not been reviewed previously in this publication. There are many important phases of spore resistance which might be reviewed and discussed. Considering all the chemical and physical influences which have been tested in some way upon the resistance and viability of bacterial spores, the literature is immense, overwhelming, and exceedingly contradictory. Any pretense to a thorough and comprehensive coverage would be presumptuous if not futile. Therefore, for the purposes of this review, consideration has been limited to what the writer considers some of the significant information with reference to the resistance of bacterial spores in relation to spore germination and its inhibition. Even with this limitation, it is impossible to cover all the relevant literature upon the subject.

Before proceeding to the specific subject matter of this review, a few general references upon the resistance of bacterial spores may be mentioned. Knaysi (1) presents an excellent and comprehensive review and evaluation of the literature upon the bacterial endospore which includes discussions of spore resistance and germination. Heat resistance has been reviewed and discussed by Esty (2), Rahn (3), and Schmidt (4). Radiation resistance has been reviewed by Lea (5) and Proctor & Goldblith (6, 7, 8). Chemical resistance has been treated extensively by Rahn (9). Many of the individual chapters in Reddish (10) contain data and considerations bearing upon spore destruction by chemicals together with extensive references to the literature. In view of the wide variety of chemical agents active to some degree in a lethal manner upon spores, it is evident that this subject does not lend itself to attempts at systematic review such as are possible with heat and radiation. The various papers appearing in the "Symposium on the Biology of Bacterial Spores" (11) also present very good reference material upon the various aspects of spore resistance.

Unfortunately perhaps for microbiologists, death of a microorganism has been defined in terms of the inability to reproduce to the point of macroscopic detection, that is the production of a colony, grossly visible growth, or a detectable metabolic product. Therefore all studies of the resistance of bacterial spores are based upon the determination of the presence of survivors following some specified treatment. This determination may be quali-

<sup>1</sup> The survey of literature pertaining to this review was completed in January, 1955.

tative in the sense of showing either growth or no growth, or may be quantitative based upon the enumeration of survivors in terms of colony count following a plating procedure. When multiple samples are examined in the qualitative or endpoint type of test, certain types of calculation may be employed to determine the number of survivors [Stumbo (12); Stumbo, Murphy & Cochrane (13); Schmidt (4)]. Most tests of radiation resistance seem to have been made in terms of the quantitative enumeration of survivors, leading to expression in terms of survivor curves. Determinations of heat resistance have been made and expressed by survivor curves, by calculation of the rate of destruction from multiple tests by the endpoint method, or qualitative tests of survival and destruction time. Resistance to chemicals has been expressed by survivor curves or from the qualitative endpoint method expressed as survival or destruction time.

It is evident that all methods presuppose the accurate and adequate recovery of survivors. Therefore, any measurement of resistance in terms of survivors may always involve two phases: (a) the actual or true survival of the spore during and following exposure to the lethal agent, and (b) the ability of the surviving spore to germinate, reproduce, and lead to a sufficient number of generations to be recognized as a survivor under the subculture conditions used.

Studies of radiation resistance do not seem to any degree to have taken into consideration the possible effect of various factors which may influence the recovery of surviving spores. In studies of chemical resistance, the greatest effort has been expended to minimize carry-over and inhibition of growth by the chemical being tested and to rigidly standardize media and conditions of subcultivation in an effort to obtain reproducible results. Only in the case of studies of heat resistance can it be said that any considerable attention has been given to the study of factors which may affect and determine the recovery of surviving spores.

Although no doubt earlier work can be cited, the discussion may suitably start from the considerations of "dormancy" of the spores of *Clostridium botulinum*. Burke (14, 15), Dickson *et al.* (16), and Dickson (17) reported extensive investigations of the "dormancy" of spores of *C. botulinum*. Extended periods of incubation were required for the recovery of survivors owing to the delayed germination of "dormant" spores. Burke (15) and Burke, Sprague & Barnes (18) contended that dormancy is inherent or normal in spores of *C. botulinum* and is further accentuated by heat injury, also that these phenomena are shown by unheated spores of *Bacillus subtilis* and *B. megatherium* in what was considered an adequate culture medium. Morrison & Rettger (19, 20) investigated the problem with cultures of a *Bacillus* species isolated from spoiled evaporated milk and also with stock strains of several species of the genus *Bacillus*. From their rather extensive investigations it was demonstrated that the composition of the subculture medium was important in determining the occurrence of dormancy and in the adequate recovery of survivors. Curran & Evans (21) further pointed out the

importance of the subculture medium in determining the survivors in resistance tests. Using spores of several species of *Bacillus* and exposure to ultraviolet light, heat, or mercuric chloride, it was amply demonstrated that very dramatic differences in the number of survivors could be obtained by the use of different plating media. The addition of cow blood or glucose to nutrient agar greatly increased the number of surviving spores detected. In some cases an infusion agar or tomato juice-milk powder agar was considerably more productive than nutrient agar. It was concluded that spores which survive drastic killing influences are more exacting in their nutritional requirements than the total population, and that enrichment is necessary for the accurate enumeration of survivors. The enrichment substances required may vary with the species of organism, the nature of the lethal factor, and the extent of the killing action. It was also shown that spores surviving destructive action and seeded into inadequate media rapidly lose their viability and cannot be recovered.

Nelson (22) studied factors which influence the growth of heat-treated bacteria, including spores of a strain of *B. subtilis*. Different basal media plus agar were not equally suitable for the recovery of heated spores, confirming the fact that the apparent survival of heat-treated spores can be varied by the use of different media.

Olsen & Scott (23), in studying the heat resistance of strains of *C. botulinum*, found that various media giving equivalent counts of unheated spores gave divergent estimates of the number of survivors following heat treatment. It was found that the addition of soluble starch markedly increased the recovery of heated spores. Wynne & Foster (24), using mildly heated spores of *C. botulinum*, obtained higher counts with the addition of starch to complex media. It was also found [Foster & Wynne (25)] that small quantities of unsaturated fatty acids which did not inhibit the growth of a vegetative inoculum inhibited germination of the spores and that this inhibition could be overcome by the addition of starch.

Olsen & Scott (26) present a detailed study of the effect of starch, charcoal, and serum albumin upon the determination of surviving spores of *C. botulinum* and other species of clostridia in pork infusion agar. It is believed that these materials adsorb inhibitory substances present in the medium. Murrell, Olsen & Scott (27) report a similar study of the effect of adsorbents upon the enumeration of heated spores of *Bacillus* species. It was found that, as the period of heating increased, the survivors became increasingly sensitive to the inhibitors present in the medium, while unheated spores showed little or no evidence of such sensitivity. Starch, charcoal, and serum albumin produce similar effects in removing the action of the inhibitory substances, most probably by adsorption. The results of a study of several different media demonstrated that all charcoal-treated media were not equally efficient in yielding the maximum number of survivors, suggesting that some media contain inhibitors not adsorbed by charcoal. It is the conclusion in both of these papers that the experiments furnish no evidence regarding the nature

of the inhibitors although it is probable that long-chain fatty acids are involved.

Amaha (28) has studied several phases of the effects of subculture media upon the recovery of survivors in heat resistance tests and upon the apparent survival time. Subcultivation in yeast extract broth, liver broth, or nutrient broth plus glucose showed a survival time for spores of *Bacillus natto* heated in phosphate buffer at 100°C. twice as long as that observed in nutrient broth. Cysteine or thioglycollate did not increase the apparent survival time when added to nutrient broth. Fructose, mannose, galactose, sucrose, maltose, soluble starch, pyruvate, or a glycerophosphate added to nutrient broth increased the apparent survival time, while arabinose, xylose, lactose, trehalose, mannitol, glycerol, glycogen, lactate, acetate, or succinate did not. Using a synthetic medium composed of 18 amino acids, nine vitamins, four purine-pyrimidine bases, and salts, it was found that a vegetative inoculum developed growth without added carbohydrate, whereas a spore inoculum did not. All of the carbon sources which gave increased survival time when added to nutrient broth supported growth of a spore inoculum in the synthetic medium. A quantitative study of the effect of the addition of glucose to nutrient broth upon the apparent survival time showed 0.01 per cent to be almost as effective as 0.5 and 0.1 per cent, and 0.005 and 0.001 per cent to show an appreciable effect.

In further studies with this synthetic medium with glucose as the source of carbon [Amaha (29)], the effect of single omission of vitamins, single addition of vitamins, or the use of single amino acids as nitrogen sources was tested. It was found that biotin was essential for development and that the further addition of thiamine and pyridoxal resulted in the maximum survival times. With single amino acids as sources of nitrogen, different apparent survival times were obtained. Glutamic acid and all amino acids which are known to be converted easily into glutamic acid gave longer survival times.

Mehl & Wynne (30) reported the rate of germination of spores of PA 3679 to be a function of the temperature over the range 20 to 45 C. and to follow the Arrhenius equation when thermolability was used as the criterion of germination. However, Williams & Reed (31) found that lower temperatures of incubation were more favorable than higher for maximum recovery of spores of *C. botulinum* when using colony counts or apparent survival times from heat processing. Sugiyama (32) also found lower temperatures of incubation to be more favorable for the recovery of survivors. Pearce & Wheaton (33) reported that the spores of a thermophilic bacillus, after heating drastically in cream style corn, autosterilized if held at 20 C., which is below the minimum growth temperature of the organism. Autosterilization was determined by reincubating thermal death time cans which had just been heated for partial destruction of the inoculum and then stored at 20 C. Autosterilization, in terms of the number of heat-treated containers in which spoilage developed following reincubation, was progressive with the storage time. Depending therefore on the properties of the organism and substrate

and the criteria used for germination, different effects of incubation temperature may be observed.

Evans & Curran (34) reported that mild pre-heating of the spores of mesophilic bacilli accelerated their subsequent germination in a nutrient substrate, the criteria of germination being thermolability, although with one exception the heating had no measurable influence on the number of spores that germinated. Subsequent studies with spores of thermotolerant and thermophilic bacilli [Curran & Evans (35)] demonstrated that sublethal heating of the spores had a very marked influence upon the number of spores that would germinate and form colonies upon a plating medium. The proportion of spores which responded to preheating (activation) was found to depend upon the amount of heating, the composition of the preheating medium, the temperature at which the spores were formed, and the temperature at which they were subcultured. Response to preheating was greater when the temperature of subcultivation was suboptimal. Preheating seems to lower the minimal temperature at which germination can occur. Curran & Evans (36) in further studies found that heat-activated spores stored in incomplete nutrient substrates either die rapidly or undergo deactivation with retention of viability and can be reactivated by post-storage heating. Activation of spores of PA 3679 by mild heating has been reported by Reynolds & Lichtenstein (37) and Stumb, Murphy & Cochrane (13).

These results might suggest that spore suspensions to be used in testing the lethal effect of chemicals or physical agents other than heat should be heat treated for activation and development of maximum viable spore count prior to the lethal treatment. Reddish (38), however, has reported studies that show that preheating of a spore suspension reduces its resistance to phenol, formaldehyde, and other chemical agents. Whether, following preheating, the preliminary stages of germination as represented by permeability change take place even in the presence of a chemical so that many of the spores assume the resistance of a vegetative cell, or whether preheating reduces resistance to chemicals by some other means, is not known. The fact that many spores require preheating in order to develop and the fact that preheating may affect sensitivity to chemicals or non-thermal physical agents pose problems in the determination of the resistance of spores to lethal agents other than heat, the immediate solution of which is not yet in view. Mefferd & Campbell (39) found activation by furfural comparable to that by heat to take place with spores of thermotolerant and thermophilic bacilli.

A number of studies have been conducted in recent years concerning the effect of antibiotics upon heat resistance and germination of bacterial spores, stimulated primarily by thoughts of the possible application of antibiotics to food processing [Curran & Evans (40, 41); Andersen & Michener (42); Adams, Ayers & Tischer (43); Bohrer (44); Burroughs & Wheaton (45); Cameron & Bohrer (46); Williams & Campbell (47); Wynne & Harrell (48); Wynne, Collier & Mehl (49); Kaufman, Ordal & El-Bisi (50, 51)].

The results have been somewhat variable and whether antibiotics have



been found to be sporocidal, sporostatic, or ineffective depends not only upon the specific antibiotic and its concentration but also upon the test organism used and the specific conditions of the test. In most cases the criterion of action upon the spores has been the failure to achieve any development of subcultures in media or natural food products following inoculation with a fairly high level of spores, with or without some mild heat treatment. In general the results would indicate either failure to exert sporostatic or sporocidal action against the total inoculum, thus resulting in growth and spoilage, even if derived from only a small fraction of the inoculum, or a temporary and at times prolonged sporostasis which results in prolonged "dormancy" followed by germination and development of some of the spores. The demonstrated ineffectiveness of antibiotics against spores of *C. botulinum* has discouraged any possible applications in the food industry. The action of antibiotics in exerting either direct or indirect sporostatic action for a prolonged period of time is, however, of interest in the general biological problem of studies of spore germination.

Fischoeder (52) found that 98 per cent of *Bacillus anthracis* spores became heat sensitive following 1 hr. incubation in broth at 37 C. while only 50 per cent reached the same state in 5 hr. in saline. Curran (53) studied the effect of osmotic pressure and nutrient concentration upon spore germination of *Bacillus mycoides*, using microscopic observation, and noted 80 to 90 per cent germination in 90 min. in 0.16 per cent peptone. A minimum concentration of peptone of .025 per cent gave 20 per cent germination in three days; below this concentration no germination occurred in three days. Knaysi (54) and Knaysi & Baker (55) demonstrated the ability of spores of *B. mycoides* to germinate in a nitrogen free medium containing a source of energy such as glucose and acetate. The germination rate was slow and only a small percentage of the total spores germinated. Since the studies were conducted for the purpose of cytological examination, no efforts were made to accelerate the rate of germination or achieve maximum percentage germination. Knaysi (56) studied in more detail some of the cytological properties of spores of *B. mycoides* germinated in glucose-acetate mixtures. Approximately 23 to 65 per cent germination was obtained in 16 hr. at 33°C. It was noted that the developed cells were gram-negative with gram-positive nuclei and highly permeable to neutral red in dilute (1 to 10 mg. per liter) solution. Also noted was the fact that a high proportion of the spores that did not develop to a vegetative cell were readily permeable to dyes in dilute solution. Aging of the suspension reduced the proportion of cells which germinated. Knaysi (1), discussing this work, stated:

Long and careful study of the minimum requirements for spore germination in strain C<sub>2</sub> of *B. mycoides* in connection with the development of vegetative cells transparent to electrons, led the reviewer to the conclusion that normal mature spores of this organism are able to germinate in a solution of glucose and sodium acetate; and that this ability is enhanced when the sugar is activated by heat, or by traces of potassium phosphate. When the spores are allowed to age, in culture or in aqueous

suspension, their ability to germinate is decreased and they gradually fail to respond to activation of the sugar by heat, or later by phosphate. These spores are "dormant" in the glucose-acetate solution, for the addition of a small quantity of yeast extract or tryptone induces many of them to germinate. Indeed, it is possible to raise, at will, the percentage of germinating spores by controlling the composition and the reduction potential of the medium.

Wynne & Foster (24, 25, 57, 58), using the heat sensitivity or "thermolability" criterion of germination, studied the factors affecting germination of the spores of *C. botulinum* and other clostridia. Germination of spores of *C. botulinum* in a complex medium, brain-heart infusion, was markedly delayed by the exclusion of all traces of CO<sub>2</sub>. Using a synthetic medium, no germination of *C. botulinum* spores was observed in 17 days when CO<sub>2</sub> was excluded although 61 per cent germination took place in five days when the atmosphere contained 1 per cent CO<sub>2</sub>. No CO<sub>2</sub> effect could be demonstrated either with four other species of clostridia or with four species of bacilli using complex organic media. It is suggested that certain CO<sub>2</sub> by-passing substances present in these media are utilizable by these organisms but not by *C. botulinum*. In the brain-heart infusion the CO<sub>2</sub> requirement of *C. botulinum* spores could be by-passed by oxalacetate. In the synthetic medium neither oxalacetate or tricarboxylic acid cycle intermediates could substitute for CO<sub>2</sub> although yeast extract could do so.

Andersen (59) reported that the addition of NaHCO<sub>3</sub> (0.10 to 0.15 per cent) to media for counting spores of *C. botulinum* was essential for the prompt and uniform development of colonies and for maximum counts to be obtained. Reynolds *et al.* (60) reported higher spore counts of PA 3679 when NaHCO<sub>3</sub> was added to a tryptone-yeast extract—thioglycollate medium. Wynne, Schmieding & Daye (61) reported a beneficial effect in increasing the spore count of *C. botulinum* 62A by the addition of NaHCO<sub>3</sub> to Yesair pork infusion medium, and a similar beneficial effect of NaHCO<sub>3</sub> for *C. botulinum* 115B in a yeast-extract glucose medium. Spore counts of several other species of *Clostridium* were not increased by the addition of NaHCO<sub>3</sub> to either medium.

Long chain fatty acids (oleic, linoleic, and linolenic) were found by Foster & Wynne (25) to inhibit germination of spores of *C. botulinum* but not the germination of spores of several aerobic species of bacilli. The inhibiting effects of the fatty acids could be relieved by the incorporation of starch in the germination medium. Roth & Halverson (62) found that unsaturated fatty acids do not inhibit spore germination unless they are rancid. Benzoyl peroxide showed similar inhibition. The effect of the rancid fatty acids could be partially overcome by catalase. Formation of colonies on solid media was used as an index of germination, and the organisms included in the study were PA 3679, *C. botulinum*, *B. subtilis* and *B. stearothermophilus*.

Hills (63) also used "thermolability" as a criterion of germination in studies of the germination of spores of *B. anthracis*. Using a gelatine hydrolysate medium plus 0.005 per cent tyrosine, which produced very slow germination,

markedly accelerated germination was obtained in the presence of 0.01 per cent fresh yeast. It was found that yeast could be replaced by adenosine at a concentration of 0.2  $\mu\text{g/ml}$ . Hills (64) studied the effect of amino acids upon the rapid germination of spores of *B. anthracis* and found L-alanine and L-tyrosine together with adenosine to greatly accelerate spore germination. The L-alanine effect was not replaceable by related compounds and was strongly inhibited by D-alanine. Tyrosine was replaceable by related compounds and activity was not inhibited by DL-tyrosine. Hills (65) determined the requirements for rapid spore germination for four strains of *B. anthracis* and one strain each of *B. cereus*, *B. megatherium* and *B. subtilis*. Phosphate buffer was the basal medium to which was added adenosine, alanine and tyrosine singly and in combinations. L-alanine was present in all cases where significant acceleration of germination took place. The other two compounds were either without effect or only effective in combination with L-alanine. In the one case tested, *B. subtilis*, the effect of L-alanine was strongly inhibited by D-alanine at a molar ratio of 1:30.

Powell (66) confirmed the effect of L-alanine in phosphate buffered glucose solution, using both loss of heat resistance and staining of the spores as criteria of germination. Correlation of the "thermolability" and staining techniques was shown. Germination was inhibited by 10 mM concentration of 8-hydroxyquinoline but not by 1 mM. BAL (2:3-dimercaptopropanol) inhibited germination completely at 10 mM concentration and this could be partially reversed by soluble salts of zinc, magnesium, copper, and iron. In this work a strain of *B. subtilis* was used as the test organism. The effect of adenosine upon rapid germination of spores of *B. subtilis* and *B. cereus* was confirmed by Pulvertaft & Haynes (67), using the loss of refractility as observed in the phase microscope as the criterion of germination. Powell (68) studied spores of a strain of *B. megatherium* which required only the addition of glucose to phosphate buffer to produce 80 to 90 per cent germination in 30 min. Equally rapid germination was found in unbuffered glucose or glucose buffered with acetate or bicarbonate. Inhibitors of respiration or glycolysis did not retard the rate of germination at the concentration tested (cyanide, azide, 2:4-dinitrophenol, fluoride, iodoacetate, oxalate, and citrate). Further correlations of the measurement of spore germination by thermolability and staining are shown and measurements of the rate of spore germination by changes in light transmission either by turbidimetric or nephelometric methods are introduced.

Powell & Strange (69) germinated spores of a culture of *B. megatherium* in several substrates for 30 to 45 min. and found a decrease in spore dry wt. of 26 to 35 per cent together with excretion of amino acids, peptides, a non-dialysable hexoseamine containing peptide, calcium, and an ultraviolet light absorbing substance. This last substance has been identified as dipicolinic acid (pyridine-2:6-dicarboxylic acid) [Powell (70)]. Calcium dipicolinate accounted for 50 per cent of the excreted solids and, therefore, for about 15 per cent of the spore dry weight. The properties of the peptide fractions ob-

tained as the exudates of germinating spores and as extracts from intact ungerminated spores have been studied by Strange & Powell (71) and Record & Grinsted (72).

Levinson & Sevag (73) studied the germination of spores of a strain of *B. megatherium* using the staining technique and respiration. The spores could be activated to give rapid germination and oxygen consumption in a substrate of salts, glucose, glutamate, and adenosine by heating for 10 min. at 60°C., or by the addition of a very low concentration of a manganese salt. A study of the effect of various inorganic ions demonstrated an inhibitory effect of high phosphate ion concentration which could be relieved or balanced by chloride or other monovalent anions, or by adenosine.

Fitz-James (74) has studied germination of spores of *B. cereus* and *B. megatherium* both cytologically and chemically. Although the primary considerations are cytological with reference to chromatin duplication, the chemical data show the changes of ribonucleic and desoxyribonucleic acid occurring during the process of spore germination.

Church, Halvorson & Halvorson (75) have determined the requirements for rapid germination of spores of six species of the genus *Bacillus*. A possible relationship between L-alanine-racemase activity [Stewart & Halvorson (76)] and germination requirements was investigated. Germination was determined by staining, thermolability, and turbidimetrically. Phosphate, adenosine and L-alanine were required for the optimum germination of *B. terminalis*, *B. cereus*, and *B. polymyxa*. Phosphate, L-alanine, and glucose were required for *B. globigii* and *B. subtilis*. *B. megatherium* showed rapid germination either in L-alanine, or in phosphate and glucose. No relation between the presence or absence of L-alanine racemase and a requirement for L-alanine for rapid germination was found. Using spores of *B. terminalis*, 90 per cent germination occurred at pH 10.5 and 73 per cent at pH 11.3 during the one hour incubation.

Wynne, Mehl & Schmeiding (77) have reported germination of spores of several *Clostridium* species in buffered glucose (pH 7.0) under anaerobic conditions. Germination was inhibited by 1 mg/ml. oleate or pH 4.8.

Mundt, Mayhew & Stewart (78) have studied germination of spores of a strain of *C. sporogenes* considered representative of those isolated from spoiled cured meat, using thermolability as the criterion of germination. It was shown that spores of this strain germinated rapidly under conditions, such as 60 per cent glucose or 8 per cent NaCl in the germination medium, which would preclude further growth. Germination was noted at a pH of 5.3 and also at a temperature of 4.4°C.

Wynne & Galyen (79, 80) have reported the rapid germination of spores of several *Clostridium* species in phosphate buffered glucose at 75°C. using thermolability as the criterion of germination.

These recent studies of spore germination show a correlation of heat lability, loss of refractility, and increased permeability as the earliest and apparently practically simultaneous changes of a spore subjected to germina-

tion conditions. However, Mefford & Wyss (81), in studies of the effect of germination of spores of *B. anthracis* upon resistance to the lethal and mutagenic effects of ultraviolet light, found a significant increase in sensitivity to both lethal effect and mutagenic action (resistance to streptomycin) before any visible changes in cytology or staining characteristics could be detected.

The sequence of events leading to the recognition of a bacterial spore as viable or dead by the usual culture procedures appears to be as follows:

Step *a*.—A change in refractility, loss of heat resistance, the occurrence of ready permeability of the spore wall to stains, together with excretion of one or more substances from the spore with accompanying loss of dry weight. Simultaneously or closely following these processes, swelling and observable cytological changes occur. This step is induced by the nutrient environment and can proceed rapidly under optimum conditions. It can take place under conditions which will not permit further cellular development, such as lack of complete nutrients, adverse pH, adverse temperature, high salt, or high sugar concentrations. It can also take place in the presence of antibiotics which prevent development of the corresponding vegetative cell. This step appears in most cases to require an energy source such as a fermentable sugar.

Step *b*.—The development of the first complete germ cell with either shedding or absorption of the spore coat. This step is somewhat continuous with step *a* but seems to be separable cytologically and to be observable subsequent to the initial changes described in step *a*.

Step *c*.—The multiplication of the germ cell through a sufficient number of generations to produce a colony on solid media, turbidity in liquid media, or a detectable metabolic product.

If a spore can initiate only step *a*, or steps *a* and *b*, it would generally not be recognized as viable and, under most conditions, would for all practical purposes be dead. Recent developments in germination studies now provide techniques whereby the separation of the steps may in some cases be distinguished and quantitatively evaluated.

In considering the nature and locus of action of adverse or lethal effects upon spores, it cannot be stated very conclusively at present whether the lethal action results in inhibition of step *a* primarily or is exhibited at steps *b* and *c*. The increased nutrient requirements of spores surviving lethal effects and the apparent increase in sensitivity of such spores to inhibitory substances in subculture media suggests that the lethal effect or locus of action may frequently be exerted by blocking steps *b* or *c*. In other cases the lethal effect may result from blocking of step *a* or altering the response of the spore to factors which call forth step *a*.

The nature of the curve relating the number (or percentage) of organisms "killed" by any lethal effect to dosage has received much consideration [Rahn (3, 9, 82, 83); Williams, Merrill & Cameron (84); Stumbe (85); Reed, Bohrer & Cameron (86); Reynolds & Lichtenstein (87); Schmidt (4)] to cite only a few of the relevant references, particularly as relating to spore destruc-

tion. The center of the dispute is the existence and validity of a logarithmic order of death, in which case the logarithms of numbers or per cent survivors plotted against dosage gives a straight line. The evidence is contradictory and both logarithmic and non-logarithmic relationships have been found.

In general, however, it may be said that the possible relationship between the factors controlling spore germination and the shape of the survivor curve found in any given experimental situation has not been adequately investigated. Using the terminology of steps *a*, *b* and *c* in which the process of germination has been described, it is apparent that the determination of a survivor curve depends upon step *c* being completed to the point of visible colony formation. It may be the case that, insofar as subculture media can affect the completion of these steps in the individual spores of a population surviving a given lethal treatment, the shape of the survivor curve may depend upon the subculture medium used for its determination. The effect could be exerted either by nutrient deficiencies or by inhibitory substances. This is not to deny that there may be other and sound biological reasons for the particular shape of any given survivor curve, but merely to point out the potential importance of factors governing spore germination or its inhibition insofar as the determination of the mathematical nature of the survivor curve is concerned.

#### ADDENDUM

The following papers have come to the attention of the author since the completion of this manuscript. Jensen (88) and Preuner (89) have applied the term "pre-vegetative" phase to the events described as step (*a*) in this review and report studies of this early stage of germination. Fitz-James (90, 91) presents detailed studies of the phosphorus fractions of spores and vegetative cells of *B. cereus* and *B. megaterium* and a correlation of the chemical and cytological changes which take place during the germination of spores of these organisms. Harrell & Halvorson (92) have attempted to analyze further the role played by L-alanine in accelerating spore germination. Perry & Foster (93) have confirmed the presence of pyridine-2,6 dicarboxylic acid in the ungerminated spores of *B. mycoides* and followed the biosynthesis of this compound during endotrophic sporulation.

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